Vascular endothelial growth factor (VEGF) induces adhesion molecules on endothelial cells during inflammation. Here we examined the mechanisms underlying VEGF-stimulated expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin in human umbilical vein endothelial cells. VEGF (20 ng/ml) increased expression of ICAM-1, VCAM-1, and E-selectin mRNAs in a time-dependent manner. These effects were significantly suppressed by Flik-1/kinase-insert domain containing receptor (KDR) antagonist and by inhibitors of phospholipase C, nuclear factor (NF)-κB, sphingosine kinase, and protein kinase C, but they were not affected by inhibitors of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) 1/2 or nitric-oxide synthase. Unexpectedly, the phosphatidylinositol (PI) 3'-kinase inhibitor wortmannin enhanced both basal and VEGF-stimulated adhesion molecule expression, whereas insulin, a PI 3'-kinase activator, suppressed both basal and VEGF-stimulated expression. Gel shift analysis revealed that VEGF-stimulated NF-κB activity. This effect was inhibited by phospholipase C, NF-κB, or protein kinase C inhibitor. VEGF increased VCAM-1 and ICAM-1 protein levels and increased leukocyte adhesiveness in a NF-κB-dependent manner. These results suggest that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNAs was mainly through NF-κB activation with PI 3'-kinase-mediated suppression, but was independent of nitric oxide and MEK. Thus, VEGF simultaneously activates two signal transduction pathways that have opposite functions in the induction of adhesion molecule expression. The existence of parallel inverse signaling implies that the induction of adhesion molecule expression by VEGF is very finely regulated.

The adhesive properties of the endothelium, the single-cell lining of the cardiovascular system, are central to its physiology and pathophysiology (1, 2). In health, the luminal endothelial cell surface is a relatively nonadhesive and nonthrombo-
 EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Recombinant human vascular endothelial growth factorα (VEGFα), placenta growth factor, and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems. Fik-1 kinase-insert domain containing receptor (KDR) antagonist SU1498, nitric-oxide synthase (NOS) inhibitor, L-arginine methyl ester (L-NAME) and its inactive isomer, N-nitro-L-arginine methyl ester (L-NNAME) were purchased from Calbiochem. PI 3'-kinase inhibitors wortmannin and LY294002 were purchased from RBI, Inc. MEK 1/2 inhibitor PD98059 was obtained from New England Biolabs. PLC inhibitor U73122 was purchased from Biomol Research Laboratory Inc. Sphingosine kinase inhibitor, N,N-dimethylsphingosine (DMS) was purchased from ICR Pharmaceuticals. NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) and protein kinase C (PKC) inhibitor chelerythrine chloride were purchased from Sigma. Media and sera were obtained from Life Technology, Inc. Functional blocking antibodies for ICAM-1 (clone P2A4), VCAM-1 (clone P3C4), and E-selectin (clone P2H3) were purchased from Chemicon, Inc. Most other biochemical reagents were purchased from Sigma, unless otherwise specified. HUVECs were prepared from human umbilical cords by collagenase digestion and maintained as described previously (18).

RNAse Protection Assay (RPA) for Expression Analysis of ICAM-1, VCAM-1, and E-selectin mRNA Transcripts—The partial cDNAs of human ICAM-1 (nucleotides 859–1225, GenBank accession NM_000201), human VCAM-1 (nucleotides 538–816, GenBank accession M60335), and human E-selectin (nucleotides 783–989, GenBank accession M30640) were amplified by polymerase chain reaction and subcloned into pBluescript II KS+(Stratagene). After linearizing with EcoRI, in vitro transcribed antisense RNA probes were synthesized in vitro transcription using T7 polymerase (Ambion Maxiscrypt kit) and gel purified. RPA was performed on total RNAs using the Ambion RPA kit. An antisense RNA probe of human cyclophilin (nucleotides 135–239, GenBank accession X52856) was used as an internal control for RNA quantification.

Electrophoretic Gel Mobility Shift Analysis—HUVECs were incubated with the indicated agents for the indicated times and then washed twice with phosphate-buffered saline. Nuclear proteins were extracted as follows. The cells were scraped into buffer A (10 mmol/liter HEPES, 1.5 mmol/liter MgCl2, 10 mmol/liter KCl) and centrifuged briefly. The cell pellet was resuspended in buffer A plus 0.1% Nonidet P-40. After centrifugation at 14,000 rpm for 10 min, the nuclear pellet was resuspended in buffer B (20 mmol/liter HEPES, 1.5 mmol/liter MgCl2, 0.4 mol/liter NaCl, 0.2 mol/liter EDTA, 25% glycerol, dithiothreitol, phenylmethylsulfonyl fluoride, and leupeptin). After centrifugation at 14,000 rpm for 10 min, the supernatant, which contains the nuclear proteins, was diluted with buffer C (20 mmol/liter HEPES, 50 mmol/liter KCl, 0.2 mmol/liter EDTA, 20% glycerol, dithiothreitol, phenylmethylsulfonyl fluoride, and leupeptin). The protein concentrations were measured using Coomassie Plus Protein Assay Reagent (Pierce). Protein binding reaction was a 30-min incubation of 10 μg of nuclear protein with a 32P-end-labeled, double-stranded oligonucleotide containing the NF-κB binding site on the human VCAM-1 promoter (5′-CTTGGAGGATTTCCCTCC-3′) (19). Cold competition controls were performed by preincubating the nuclear proteins with unlabeled 20-fold molar excess of the NF-κB double-stranded oligonucleotide for 20 min before the addition of the 32P-labeled oligonucleotide. As a negative control, a cold competition was also performed with an irrelevant octamer transcription factor (Oct-1) oligonucleotide (5′-TAGAGGATCCATGCAATGCGGGGATCC-3′). In antibody supershift experiments, nuclear extracts were incubated for 30 min at room temperature with 2 μg of polyclonal rabbit antibodies to human NF-κB proteins (p65, p50, p52, RelB, and c-Rel; Santa Cruz Biotechnology) and then incubated with labeled oligonucleotide. The mixtures were resolved on native 5% polyacrylamide gels, which were dried and autoradiographed.

Western Blot Analysis—For Western blot analysis, samples were mixed with sample buffer, boiled for 10 min, separated by SDS-polyacrylamide gel electrophoresis under denaturing conditions, and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were washed with labeled oligonucleotide. The mixtures were resolved on native 5% polyacrylamide gels, which were dried and autoradiographed.

NOS Activity—HUVECs were cultured in 24-well plates. At confluence, the medium was replaced with medium without phenol red in the presence or absence of VEGF, l-NAME, and p-NAME. After a 30-min incubation, this medium was collected, and total NO was measured with a nitrate/nitrite colorimetric assay kit (Cayman Chemical) according to the manufacturer’s instruction. The measured value was normalized to the number of HUVECs in the well from which the medium was collected.

Flow Cytometry Analysis—HUVECs were stimulated with VEGF or TNF-α for 8 h. Then, cells were washed twice with cold phosphate-buffered saline, removed by careful trypsinization, and washed again with Ca2+/Mg2+-free phosphate-buffered saline before incubating with 20% fetal bovine serum for 30 min. After two washes, cells were incubated with an antibody against human VCAM-1 or ICAM-1 (Santa Cruz Biotechnology) for 1 h at 4 °C. Cells were then washed twice with phosphate-buffered saline/fetal bovine serum and incubated for 1 h at 4 °C with a fluorescein isothiocyanate-conjugated secondary antibody. Cells were then fixed with 2% paraformaldehyde and analyzed by flow cytometry in a fluorescence-activated cell sorter cytoflurometer (Becton Dickinson). The results were gated for mean fluorescence intensity above the fluorescence produced by the secondary antibody alone.

Adhesion Assay—Leukocyte-endothelial adhesion was measured by fluorescent labeling of leukocytes according to the methods of Akeson and Woods (20). Peripheral blood leukocytes were separated from hepar-
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RESULTS

VEGF Increased Expression of ICAM-1, VCAM-1, and E-selectin mRNAs in HUVECs—We developed a method of RPA by which we can detect the mRNA levels of ICAM-1, VCAM-1, E-selectin, and cyclophilin simultaneously. The addition of 20 ng/ml VEGF increased the expression of ICAM-1, VCAM-1, and E-selectin mRNAs as early as 2 h and produced a maximal effect at 4 h (Fig. 1, A and B). The higher expression levels declined thereafter, but the level of ICAM-1 and VCAM-1 continued to be higher than control for up to 8 h. The maximum mean increases in ICAM-1, VCAM-1, and E-selectin were 5.2-, 9.8-, and 2.2-fold, respectively (Fig. 1B). As a positive control, the addition of 1 ng/ml TNF-α for 1 h also markedly increased the expression of ICAM-1, VCAM-1, and E-selectin (Fig. 1A).

Inhibitors Changed VEGF-stimulated Expression of ICAM-1, VCAM-1, and E-selectin mRNAs—To examine the receptor/second messenger mechanisms leading to induction of adhesion molecules by VEGF, a receptor antagonist and various intracellular kinase inhibitors were added to 20 ng/ml VEGF-treated HUVECs. A specific KDR antagonist (SU1498, 20 μM)
completely inhibited VEGF-stimulated expression of the adhesion molecule mRNAs (Fig. 2, A and B). Placenta growth factor is known to be a specific Flt-1 ligand (21). 10–500 ng/ml placenta growth factor did not produce any effect on expression of the adhesion molecules (data not shown). MEK 1/2 inhibitor (PD98059, 50 μM) did not produce any changes, whereas PLC inhibitor (U73122, 1 μM), NF-κB inhibitor (PDTC, 50 μg/ml), sphingosine kinase inhibitor (DMS, 5 μM), and PKC inhibitor (chelerythrine chloride, 5 μM) suppressed VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin (Fig. 2, A and B). Unexpectedly, the PI 3-kinase inhibitor (wortmannin, 30 nM) enhanced VEGF-induced expression of the three adhesion molecule mRNAs (Fig. 2, A and B). These results suggested that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNAs may be mediated mainly through activation of PLC and NF-κB, along with PI 3-kinase-mediated suppression. The process appears to be independent of the MEK/ERK pathway.

VEGF-induced Expression of ICAM-1, VCAM-1, and E-selectin Was Correlated with NF-κB Activity—Because the expression of adhesion molecules is mainly regulated by NF-κB (22–24), we examined NF-κB activity in HUVECs treated with VEGF in the absence or presence of various intercellular kinase inhibitors. The addition of 20 ng/ml VEGF increased NF-κB activity as early as 0.5 h and produced a maximal effect at 1 h (Fig. 3, A and D). These effects declined but continued to be higher than control levels up to 6 h. The maximum mean increase in NF-κB activity was 5.8-fold. As a positive control, the addition of 1 ng/ml TNF-α for 1 h increased NF-κB activity.

A 20-fold molar excess of unlabeled competitor almost completely blocked the NF-κB binding site, whereas the irrelevant oligonucleotide, Oct-1, did not produce any effect on the binding.
The MEK 1/2 inhibitor (PD98059, 50 μM) did not produce any change in VEGF-induced NF-κB activity, whereas KDR antagonist (SU1498, 20 μM), PLC inhibitor (U73122, 1 μM), NF-κB inhibitor (PDTC, 50 μg/ml), sphingosine kinase inhibitor (DMS, 5 μM), and PKC inhibitor (chelerythrine chloride, 5 μM) suppressed VEGF-induced NF-κB activity (Fig. 3, B and D). PI 3-kinase inhibitor (wortmannin, 30 nM) enhanced VEGF-induced NF-κB activity (Fig. 3, B and D). Overall, VEGF-induced NF-κB activity was correlated with the expression of adhesion molecules by VEGF. We performed supershift experiments using specific antibodies to p65 (RelA), RelB, c-Rel, p50, and p52 to reveal the identities of the proteins in the VEGF-induced NF-κB binding complex. Incubation with antibody to p65 or p50, but not with antibody to RelB, c-Rel, or p52, shifted the protein-DNA complexes (Fig. 3C). These data indicate that VEGF activates NF-κB in the form of a p65/p50 heterodimer in HUVECs.

VEGF-induced Expression of ICAM-1, VCAM-1, and E-selectin Was Independent of NO but Was Suppressed by Activation of PI 3'-Kinase—The addition of NOS inhibitor L-NAME (3 mM), but not its inactive D isomer D-NAME (3 mM), markedly suppressed basal and VEGF-stimulated NOS activity (Table I). Under these conditions, both the basal and the VEGF-stimulated expression of the three adhesion molecules was enhanced (Fig. 4, A and B). Inhibition of PI 3'-kinase activity with 100 nM LY294002 produced a similar effect (data not shown). Alternatively, activation of PI 3'-kinase with 50 microunits of insulin suppressed basal and VEGF-stimulated expression of the three adhesion molecules (Fig. 4, A and B).

VEGF Increased the Protein Levels of ICAM-1 and VCAM-1, and Inhibitors Changed This Effect—Because ICAM-1 and VCAM-1 showed the strongest response to VEGF among the three molecules we examined, we looked further at the protein levels of ICAM-1 and VCAM-1 in HUVECs treated with VEGF. The addition of 20 ng/ml VEGF increased protein levels of ICAM-1 as early as 2 h and produced a maximal effect at 4–6 h (Fig. 5A, upper panels). These effects declined but continued to be higher than control levels up to 12 h. The maximum mean increase in ICAM-1 was 6.5-fold. 1 ng/ml TNF-α, used as a positive control, increased protein levels of ICAM-1 and VCAM-1 markedly at 6 h. The effect of various inhibitors on VEGF-induced protein levels of ICAM-1, VCAM-1, and E-selectin was not changed (Fig. 4, A and B). The addition of PI 3'-kinase inhibitor wortmannin (30 nM) markedly suppressed basal and VEGF-stimulated NOS activity (Table I). Under these conditions, both the basal and the VEGF-stimulated expression of the three adhesion molecules was enhanced (Fig. 4, A and B). Inhibition of PI 3'-kinase activity with 100 nM LY294002 produced a similar effect (data not shown). Alternatively, activation of PI 3'-kinase with 50 microunits of insulin suppressed basal and VEGF-stimulated expression of the three adhesion molecules (Fig. 4, A and B).
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Fig. 7. Second messenger pathways in VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin in endothelial cells. After the binding of VEGF to the VEGF receptor (VEGFR, Flk-1/KDR), PLCγ, PI 3'-kinase, and MEK/ERK were activated. NF-xB was activated through activation of PLCγ-phosphoinositide kinase-PKC cascade. This cascade is the main pathway that induces the transcription of ICAM-1, VCAM-1, and E-selectin. Unidentified pathways through activation PI 3'-kinase or PI 3'-kinase/Akt may suppress the transcription of ICAM-1, VCAM-1, and E-selectin. The induction of NO and the activation of MEK/ERK by VEGF may not be involved in the regulation of ICAM-1, VCAM-1, and E-selectin expression.

ICAM-1 and VCAM-1 was similar to their effect on VEGF-induced mRNA levels. MEK 1/2 inhibitor did not produce any changes, whereas inhibitors of PLC, NF-xB, and PKC suppressed VEGF-induced protein levels of ICAM-1 and VCAM-1 (Fig. 5B). PI 3'-kinase inhibitor enhanced VEGF-induced protein levels of ICAM-1 and VCAM-1 (Fig. 5B). Using flow cytometry, we confirmed that the protein levels of VCAM-1 and ICAM-1 on the endothelial cell surface increased after treatment of 20 ng/ml VEGF for 6 h (data not shown).

VEGF-induced Leukocyte Adhesiveness Was Correlated with VEGF-induced Expression of Adhesion Molecules—Because the induction of adhesion molecules in endothelial cells induces leukocyte adhesiveness, we examined whether VEGF induces leukocyte adhesion to HUVECs. Accordingly, the addition of 20 ng/ml VEGF produced ~3.1-fold increases in leukocyte adhesiveness after 6 h compared with the addition of control buffer (Fig. 6, A and B). The Flk-1/KDR antagonist (SU1498, 20 μM), MEK 1/2 inhibitor (PD98059, 50 μM) PLC inhibitor (U73122, 1 μM), NF-xB inhibitor (PDTC, 50 μM/ml), and PKC inhibitor (chelerythrine chloride, 5 μM) all suppressed basal and VEGF-induced leukocyte adhesiveness (Fig. 6, A and B). However, the PI 3'-kinase inhibitor (wortmannin, 30 nM) produced a profoundly variable effect on VEGF-induced leukocyte adhesiveness (Fig. 6, A and B). Although functional blocking antibodies to ICAM-1, VCAM-1, and E-selectin did not produce significant changes in basal leukocyte adhesiveness, they reduced VEGF-induced leukocyte adhesiveness (Fig. 6, A and B). A triple combination of these antibodies produced marked suppression of VEGF-induced leukocyte adhesiveness (Fig. 6, A and B).

DISCUSSION

VEGF exerts its action by binding to two cell surface receptors, Flk-1/KDR and Flt-1 (25). In Flk-1/KDR null mutant mice, development of endothelial and hematopoietic cells is impaired (26). Flt-1 null mutant mice have an apparent overgrowth of endothelial cells, accompanied by blood vessel disorganization (27). The distinct phenotypes of the Flk-1/KDR and Flt-1 knockout animals show that these receptors have different biological functions. Therefore, it is likely that the two VEGF receptors signal through different transduction pathways. Our results indicate that a specific Flk-1/KDR antagonist completely blocked VEGF-induced expression of ICAM-1, VCAM-1, and E-selectin and blocked VEGF-induced NF-xB activity. However, a specific Flt-1 ligand, placenta growth factor, did not produce any effect on the expression of the adhesion molecules. Thus, VEGF-induced expression of adhesion proteins in endothelial cells occurs through VEGF binding to the Flk-1/KDR receptor, but not to the Flt-1 receptor (Fig. 7).

Upon activation of the Flk-1/KDR receptor in endothelial cells, three major second messenger pathways elicit cell proliferation, migration, survival, and NO production (7–17). These pathways are: PI 3'-kinase-serine-threonine protein kinase/Akt cascade, the tyrosine phosphorylation of PLCγ, and the MEK/ERK cascade (7–17). VEGF-induced activation of PI 3'-kinase results in phosphorylation of Akt in endothelial cells (9, 14, 15). This phosphorylated Akt results in phosphorylation of Bad and endothelial NOS, resulting in cell survival, NO production and migration (9, 14, 15, 17). Pharmacological inhibition of PI 3'-kinase with wortmannin and LY294002 completely inhibited these VEGF-induced cellular effects in endothelial cells (9, 14, 15). Consistent with previous reports (14, 15), we found that pharmacological inhibition of PI 3'-kinase with wortmannin and LY294002 inhibited basal and VEGF-induced NO production. However, unexpectedly, our data indicated that under PI 3'-kinase inhibition, the basal expression levels of ICAM-1, VCAM-1, and E-selectin mRNA were higher. Furthermore, under PI 3'-kinase inhibition, VEGF-induced expression levels were higher. Alternatively, insulin, an activator of PI 3'-kinase, decreased basal and VEGF-induced ICAM-1, VCAM-1, and E-selectin expression. These data strongly suggest that PI 3'-kinase could be an intracellular suppressor for the expression of ICAM-1, VCAM-1, and E-selectin through yet unidentified signaling pathways (Fig. 7). To our knowledge, these results are the first to demonstrate an additional role of PI 3'-kinase in suppressing the expression of adhesion molecules. Thus, selective activation of PI 3'-kinase suppresses the induction of ICAM-1, VCAM-1, and E-selectin in endothelial cells. Therefore, PI 3'-kinase may decrease inflammatory responses, and a selective activator of PI 3'-kinase could be considered as a therapeutic agent for reducing a VEGF-induced inflammation in endothelial cells.

A previous report indicated that VEGF induces expression of monocyte chemoattractant protein-1, a chemokine that is involved in recruiting leukocytes to sites of inflammation, mainly through activation of NF-xB and AP-1 in retinal endothelial cells (28). The MEK/ERK system is not involved in VEGF-induced activation of NF-xB, but it is involved in VEGF-induced activation of AP-1 in the VEGF-induced expression of monocyte chemoattractant protein-1 (28). VEGF/Flk-1/KDR binding triggers a signaling cascade that results in tyrosine phosphorylation of PLCγ (7, 11, 13). Phosphorylation of PLCγ increases intracellular levels of inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate elevates intracellular calcium through an influx from the endoplasmic reticulum. The increase in intracellular calcium also can activate sphingosine kinase to produce sphingosine 1-phosphate (29). In turn, the increase in intracellular sphingosine 1-phosphate activates PKC. In addition, activated PLCγ also activates PKC by increasing diacylglycerol. Activated PKC is known to be a strong activator of NF-xB (30). There is ample evidence that activation of NF-xB stimulates expression of ICAM-1, VCAM-1, and E-selectin mRNAs in endothelial cells (22–24). Thus, VEGF-induced activation of PLCγ and PKC is an essential step for...
induction of these adhesion molecule mRNAs in endothelial cells, and the induction occurs through NF-κB activation (Fig. 7). Upon activation of the Flk-1/KDR receptor, increased intracellular calcium and the activation of PKC or Akt result in activation of endothelial NOS and thus increased production of NO (11, 14–16). Although previous reports (31, 32) indicate that NO modulates the protein levels of VCAM-1 or ICAM-1 differently in endothelial cells, our results indicated that NO is not involved in VEGF-induced mRNA expression of ICAM-1, VCAM-1, and E-selectin. Thus, NO may modulate expression of ICAM-1 and VCAM-1 at the translational level but not at the transcriptional level. Upon activation of Flk-1/KDR, MEK/ERK signal messenger transduction pathways are activated and lead to cellular proliferation (10, 12, 13). Pharmacological inhibition of MEK/ERK pathways with PD98059 did not have any effect on the expression of ICAM-1, VCAM-1, and E-selectin mRNAs. Thus, NO and the MEK/ERK system are not involved in VEGF-stimulated expression of adhesion molecules (Fig. 7).

Induction of adhesion molecules is an initial step in inflammation mediated by leukocyte adhesion. Previous reports have shown that VEGF does not affect the expression of ICAM-1 and VCAM-1 in human dermal microvascular endothelial cells (33), whereas VEGF increased the expression of ICAM-1, but not VCAM-1 and E-selectin, in vivo in retinal capillary endothelial cells (34). Our results indicate that VEGF increased the expression of ICAM-1, VCAM-1, and E-selectin in HUVECs. Endothelial cells from different areas have different characteristics and different responses to growth factors (35, 36). Thus, the expression of adhesion molecules in response to VEGF may be different between large vessel endothelial cells and microvascular endothelial cells. Our results clearly indicated that VEGF increased VCAM-1 and ICAM-1 protein in a time-dependent manner. Accordingly, VEGF increased leukocyte adhesion in endothelial cells. Leukocyte adhesion to endothelial cells requires multiple cellular steps and intracellular second messenger signaling systems. Although the kinase inhibitors used in this study could be involved in multiple downstream effects in the response of the endothelial cells to VEGF, there were close relationships between induction of adhesion molecules and leukocyte adhesiveness. In addition, a combination of specific blocking antibodies to ICAM-1, VCAM-1, and E-selectin significantly inhibited VEGF-induced leukocyte adhesiveness to endothelial cells. Thus, VEGF-induced adhesion molecules in endothelial cells is closely involved in VEGF-induced leukocyte adhesiveness.

In summary, the present results explain how VEGF stimulates the expression of adhesion molecules in HUVECs. Our results show that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNAs was mainly through activation of PLCγ and NF-κB. The induction was suppressed by a PI 3’-kinase-mediated pathway but was independent of NO and MEK/ERK. Thus, VEGF simultaneously activates two signal transduction pathways that have opposite functions in the induction of adhesion molecule expression.

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