VEGF Stimulates Expression of ICAM-1, VCAM-1 and E-Selectin through Nuclear Factor-κB Activation in Endothelial Cells

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Summary

VEGF induces adhesion molecules on endothelial cells during inflammation. Here we examined the mechanisms underlying VEGF-stimulated expression of ICAM-1, VCAM-1 and E-selectin in HUVECs. 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 Flk-1/KDR antagonist, and by inhibitors of PLC, NF-κB, sphingosine kinase and PKC, but were not affected by inhibitors of MEK 1/2 or NOS. Unexpectedly, PI 3’-kinase inhibitor wortmannin enhanced both basal and VEGF-stimulated adhesion molecule expression, while 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 PLC, NF-κB, or PKC 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 NO 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.
Introduction

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 nonthrombogenic conduit for the cellular and macromolecular constituents of the blood. The extracellular matrix holds the basal endothelial cell surface in a well-arranged array. In certain diseases, various adhesive interactions between endothelial cells and the constituents of the blood or extracellular matrix are changed. These diseases include inflammation, atherosclerosis, pathologic angiogenesis, and vascular injury. During these disease processes, adhesion molecules are closely involved (1,2). To date, four families of cell adhesion molecules have been described: integrins, immunoglobulin superfamily members, cadherins and selectins. Members of each family have been detected in blood vessels during angiogenesis and inflammation (3).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose activities include endothelial cell survival, proliferation, migration, and tube formation (4). VEGF also acts as a proinflammatory cytokine by increasing endothelial permeability and inducing adhesion molecules that bind leukocytes to endothelial cells (5,6). The distinct signal transduction mechanisms by which VEGF induces survival, proliferation, migration, and nitric oxide (NO) production in endothelial cells have been identified (7-17). However, the signal transduction mechanisms leading to the induction of adhesion molecules are little known.
In this study, we examined signal transduction mechanisms by which VEGF induces adhesion molecules in human umbilical vein endothelial cells (HUVECs). We demonstrate that VEGF-stimulated expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin mRNAs is mediated mainly through nuclear factor-κB (NF-κB) activation with phosphatidylinositol (PI) 3’-kinase-mediated suppression, but is independent of NO and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK).
Experimental Procedures

Materials and Cell Culture

Recombinant human vascular endothelial growth factor (VEGF), placenta growth factor (PlGF), and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems. Flk-1/kinase-insert domain containing receptor (KDR) antagonist SU1498, nitric oxide synthase (NOS) inhibitor, N^G^-nitro-L-arginine methyl ester (L-NAME) and its inactive isomer, N^G^-nitro-D-arginine methyl ester (D-NAME) were purchased from Calbiochem, Inc. 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 ICN 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 No. P2A4), VCAM-1 (clone No. P3C4), and E-selectin (clone No. 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 previously described (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 PCR and subcloned into pBluescript II KS+ (Stratagene). After linearizing with EcoRI, ^32^P-labeled antisense RNA probes were synthesized by *in vitro* transcription using T7 polymerase (Ambion Maxiscript 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 PBS. Nuclear proteins were extracted as follows. The cells were scraped into buffer A (10 mmol/L HEPES, 1.5 mmol/L MgCl₂, 10 mmol/L 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 minutes, the nuclear pellet was resuspended in buffer B (20 mmol/L HEPES, 1.5 mmol/L MgCl₂, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, DDT, PMSF, and leupeptin). After centrifugation at 14,000 rpm for 10 minutes, the supernatant, which contains the nuclear proteins, was diluted with buffer C (20 mmol/L HEPES, 50 mmol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, DTT, PMSF, and leupeptin). The protein concentrations were measured using Coomassie Plus Protein Assay Reagent (Pierce). The binding reaction was a 30-min incubation of 10 μg of nuclear protein with a ^32^P end-labeled, double-stranded oligonucleotide containing the NF-κB binding site on the human VCAM-1 promoter (5’-CCTTGAAGGGATTTCCCTCC-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 minutes before the addition of the $^{32}$P-labeled oligonucleotide. As a negative control, a cold competition was also performed with an irrelevant octamer transcription factor (Oct)-1 oligonucleotide (5’-TAGAGGATCCATGCAAATGCCGGGTACC-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), 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions, and electro-blotted to nitrocellulose membranes. The nitrocellulose membranes were blocked by incubation in blocking buffer, incubated with anti-VCAM-1 polyclonal antibody (Santa Cruz Biotechnology) or anti-ICAM-1 monoclonal antibody (Santa Cruz Biotechnology), washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection according to the manufacturer's protocol (Amersham, Buckinghamshire, UK). The membrane was re-blotted with anti-actin antibody to verify equal loading of protein in each lane.

**NOS Activity**

HUVECs were cultured in 24-well plates. At sub-confluence, the medium was
replaced with medium without phenol red in the presence or absence of VEGF, L-NAME, and D-NAME. After 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 PBS, removed by careful trypsinization, and washed again with Ca²⁺/Mg²⁺-free PBS before incubating with 20% FBS for 30 min. Following two washes, cells were incubated with an antibody against human VCAM-1 or ICAM-1 (Santa Cruz Biotechnology) for 1 hr at 4°C. Cells were then washed twice with PBS/FBS and incubated for 1 hr at 4°C with a FITC-conjugated secondary antibody. Cells were then fixed with 2% paraformaldehyde, and analyzed by flow cytometry in a FACS cytofluorometer (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 Wood (20). Peripheral blood leukocytes were separated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density gradient centrifugation. The cells were labeled with Vybrant DiD (5 µM, 20 min, 37°C, Molecular Probes) in phenol red-free RPMI containing 5% FBS. The viability after labeling was always >95% as judged by trypan blue exclusion test. Cells were
washed twice and resuspended in adhesion medium (RPMI containing 2% FBS and 20 mM HEPES). The leukocytes were added (1.5 x 10^6/ml, 200 µl/well) to confluent monolayers of HUVECs that had been grown in 24-well plates and treated with various reagents and blocking antibodies. The amount of labeled cells added was assessed by recording the fluorescence signal (total signal) using a fluorescence spectrometer equipped with a microplate reader (Molecular Device). After incubation for 60 min at 37°C, non-adherent cells were removed by washing four times with pre-warmed RPMI. The fluorescent signal was reassessed by the microplate reader (adherent signal). The percentage of leukocytes adhering to HUVECs was calculated by the formula: % adherence = (adherent signal/total signal) x 100.

**Densitometric Analyses and Statistics**

All signals were visualized and analyzed by densitometric scanning (LAS-1000, Fuji Film, Tokyo). Data are expressed as mean ± standard deviation. Statistical significance was tested using 1-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at p<0.05.
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. Addition of VEGF (20 ng/ml) increased the expression of ICAM-1, VCAM-1, and E-selectin mRNAs as early as 2 hr and produced a maximal effect at 4 hr (Figures 1A and 1B). 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 hr. The maximum mean increases in ICAM-1, VCAM-1, and E-selectin were 5.2-, 9.8-, and 2.2-fold, respectively (Figure 1B). As a positive control, addition of TNF-α (1 ng/ml) for 1 hr also markedly increased the expression of ICAM-1, VCAM-1, and E-selectin (Figure 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 VEGF (20 ng/ml)-treated HUVECs. A specific KDR antagonist (SU1498, 20 μM) completely inhibited VEGF-stimulated expression of the adhesion molecule mRNAs (Figures 2A and 2B). PlGF is known to be a specific Flt-1 ligand (21). PlGF (10-500 ng/ml) 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 (Figures 2A and 2B). Unexpectedly, PI 3'-kinase inhibitor (wortmannin, 30 nM) enhanced VEGF-induced expression of the three adhesion molecule mRNAs (Figures 2A and 2B). These results suggested that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNAs may be mediated mainly through activation of PLCγ 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 intracellular kinase inhibitors. Addition of VEGF (20 ng/ml) increased NF-κB activity as early as 0.5 hr and produced a maximal effect at 1 hr (Figures 3A and 3D). These effects declined but continued to be higher than control levels up to 6 hr. The maximum mean increase in NF-κB activity was 5.8-fold. As a positive control, addition of TNF-α (1 ng/ml) for 1 hr 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 site. 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.
(Figures 3B and 3D). PI 3'-kinase inhibitor (wortmannin, 30 nM) enhanced VEGF-induced NF-κB activity (Figures 3B and 3D). 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 in order 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 (Figure 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**

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 1). Under these conditions, basal and VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin was not changed (Figures 4A and 4B). Addition of PI 3′-kinase inhibitor wortmannin (30 nM) markedly suppressed basal and VEGF-stimulated NOS activity (Table 1). Under these conditions, both the basal and the VEGF-stimulated expression of the three adhesion molecules was enhanced (Figures 4A and 4B). Inhibition of PI 3′-kinase activity with LY294002 (100 nM) produced a similar effect (data not shown). Alternatively, activation of PI 3′-kinase with insulin (50 μU) suppressed basal and VEGF-stimulated expression of the three adhesion molecules (Figures 4A and 4B).

**VEGF increased the protein levels of ICAM-1 and VCAM-1, and inhibitors changed this effect**
Because ICAM-1 and VCAM-1 showed 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. Addition of VEGF (20 ng/ml) increased protein levels of ICAM-1 as early as 2 hr and produced a maximal effect at 6-12 hr (Figure 5A, upper panels). These effects declined but continued to be higher than control levels up to 18 hr. The maximum mean increase in ICAM-1 was 8.7-fold. Addition of VEGF (20 ng/ml) increased protein levels of VCAM-1 as early as 2 hr and produced a maximal effect at 4-6 hr (Figure 5A, lower panels). These effects declined but continued to be higher than control levels up to 12 hr. The maximum mean increase in VCAM-1 was 6.5-fold. TNF-α (1 ng/ml), used as a positive control, increased protein levels of ICAM-1 and VCAM-1 markedly at 6 hr. The effect of various inhibitors on VEGF-induced protein levels of 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-κB and PKC suppressed VEGF-induced protein levels of ICAM-1 and VCAM-1 (Figure 5B). PI 3’-kinase inhibitor enhanced VEGF-induced protein levels of ICAM-1 and VCAM-1 (Figure 5B). Using flow cytometry, we also confirmed that the protein levels of VCAM-1 and ICAM-1 on the endothelial cell surface increased after treatment of VEGF (20 ng/ml) for 8 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, addition of VEGF (20 ng/ml) produced approximately 3.1-fold increases
in leukocyte adhesiveness after 8 hr compared to addition of control buffer (Figures 6A and 6B). Flk-1/KDR antagonist (SU1498, 20 µM), MEK 1/2 inhibitor (PD98059, 50 µM) PLC inhibitor (U73122, 1 µM), NF-κB inhibitor (PDTC, 50 µg/ml), and PKC inhibitor (chelerythrine chloride, 5 µM) all suppressed basal and VEGF-induced leukocyte adhesiveness (Figures 6A and 6B). However, PI 3-kinase inhibitor (wortmannin, 30 nM) produced a profoundly variable effect on VEGF-induced leukocyte adhesiveness (Figures 6A and 6B). 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 (Figures 6A and 6B). A triple combination of these antibodies produced marked suppression of VEGF-induced leukocyte adhesiveness (Figures 6A and 6B).
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-κB activity. However, a specific Flt-1 ligand, PlGF, 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 (Figure 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 MEK/ERK cascade (7-17). VEGF-induced activation of PI 3’-kinase results in phosphorylation of Akt in endothelial cells (9,14,15,17). This phosphorylated Akt results in phosphorylation of Bad and eNOS, 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 (Figure 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. Therefore, 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-κB and AP-1 in retinal endothelial cells (28). The MEK/ERK system are not involved in VEGF-induced activation of NF-κB, but they are 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 phospholipase Cγ
Phosphorylation of phospholipase Cγ increases intracellular levels of inositol 1,4,5-triphosphate (1,4,5-IP3) and diacylglycerol. 1,4,5-IP3 elevates intracellular calcium through an efflux from 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 phospholipase Cγ also activates PKC by increasing diacylglycerol. Activated PKC is known to be a strong activator of NF-κB (30). There is ample evidence that activation of NF-κB stimulates expression of ICAM-1, VCAM-1, and E-selectin mRNAs in endothelial cells (22-24). Thus, VEGF-induced activation of PLCγ and PKC are essential steps for induction of these adhesion molecules mRNAs in endothelial cells, and the induction occurs through NF-κB activation (Figure 7). Upon activation of the Flk-1/KDR receptor, increased intracellular calcium and the activation of PKC or Akt result in activation result in activation of eNOS 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 (Figure 7).
Induction of adhesion molecules is an initial step in inflammation mediated by leukocyte adhesion. Previous reports have shown that VEGF did not effect the expression of ICAM-1 and VCAM-1 in human dermal microvascular endothelial cells (33), while 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 endothelial cells’ response 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 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.
References

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Footnotes

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Abbreviations: VEGF, vascular endothelial growth factor; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; NF-κB, nuclear factor-κB; PI 3'-kinase, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; TNF-α, tumor necrosis factor-α; KDR, kinase-insert domain containing receptor; NOS, nitric oxide synthase; L-NAME, N^6^-nitro-L-arginine methyl ester; D-NAME, N^6^-nitro-D-arginine methyl ester; DMS, N,N-dimethylsphingosine; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; RPA, RNase protection assay; FBS, fetal bovine serum
Figure Legends

**Figure 1.** RPA of adhesion molecule mRNAs in VEGF-stimulated HUVECs. A, HUVECs were incubated with VEGF$_{165}$ (20 ng/ml) for the indicated times. Total RNAs (10 µg) were subjected to multiplex RPA probed with antisense ICAM-1, antisense VCAM-1, and antisense E-selectin RNA probes. Equivalent loading was confirmed by probing the same reactions with an antisense cyclophilin RNA probe (105 bp). To clarify the identity of the bands, ICAM-1 (I), VCAM-1 (V), and E-selectin (E) probes were applied individually to the total RNA from HUVECs treated with VEGF for 4 hr to reveal protected bands of 367, 279, and 187 bp, respectively. The positive control was total RNA (2 µg) from HUVECs that had been incubated with TNF-α (T, 1 ng/ml) for 1 hr and subjected to the same assay conditions. B, Densitometric analyses are presented as the relative ratio of ICAM-1, VCAM-1, or E-selectin mRNA to cyclophilin mRNA. The relative ratio measured at time 0 hr is arbitrarily presented as 1. Results were similar in three independent experiments. Bars represent the mean ± S.D. from three experiments. *, p<0.05 versus time 0.

**Figure 2.** RPA of adhesion molecule mRNAs in VEGF-stimulated HUVECs co-treated with inhibitors. A, HUVECs were incubated with VEGF$_{165}$ (20 ng/ml) for 4 hr in the presence of control buffer (CB), SU1498 (SU, 20 µM), wortmannin (WT, 30 nM), PD98059 (PD, 50 µM), U73122 (U7, 1 µM), PDTC (PT, 50 µg/ml), DMS (DM, 5 µM), or chelerythrine chloride (CC, 5 µM). Total RNAs (10 µg) isolated from the cells were subjected to RPA as described in Figure 1. B, Densitometric analyses are presented as the relative ratio of ICAM-1, VCAM-1, or E-selectin mRNA to cyclophilin mRNA. The
relative ratio measured after addition of control buffer is arbitrarily presented as 1. Results were similar in three independent experiments. Bars represent the mean ± S.D. from three experiments. *, p<0.05 versus CB plus VEGF (20 ng/ml).

**Figure 3.** Activation of NF-κB binding by VEGF and TNF-α in HUVECs. **A,** HUVECs were incubated with VEGF$_{165}$ (20 ng/ml) and TNF-α (1 ng/ml) for the indicated hours. Nuclear extracts were incubated in the absence or presence of a 20-fold molar excess of cold human VCAM-1 NF-κB oligonucleotide (lane CE) or cold Oct-1 oligonucleotide (lane O1) before the addition of radiolabeled human VCAM-1 NF-κB oligonucleotide. **B,** HUVECs were incubated with VEGF$_{165}$ (20 ng/ml) for 1.5 hr in the presence of control buffer (CB) or inhibitors, as described in Figure 2. NF-κB binding activities in nuclear extracts were assayed by gel shift assay. **C,** Nuclear extracts from HUVECs treated with VEGF$_{165}$ (20 ng/ml) for 1.5 hr were incubated with control buffer (CB), antibody specific for p65 (65), RelB (Rb), c-Rel (cR), p50 (50), or p52 (52), followed by the addition of radiolabeled human VCAM-1 NF-κB oligonucleotide. p65/p50, p65/p50 heterodimer; SS, supershift band; NS, non-specific bands. **D,** Densitometric analyses are presented as the relative ratio of NF-κB activity. The relative ratio measured at time 0 hr or from addition of control buffer is arbitrarily presented as 1. Results were similar in three independent experiments. Bars represent the mean ± S.D. from three experiments. *, p<0.05 versus time 0 or CB. #, p<0.05 versus CB plus VEGF (20 ng/ml).

**Figure 4.** RPA of adhesion molecule mRNAs in VEGF-stimulated HUVECs co-treated with NOS inhibitor, PI 3-kinase inhibitor, and insulin. **A,** HUVECs were incubated
with VEGF<sub>165</sub> (20 ng/ml) for 4 hr in the absence or presence of L-NAME (3 mM), D-NAME (3 mM), insulin (50 µU) or wortmannin (WT, 30 nM). Total RNAs (10 µg) isolated from the cells were subjected to RPA as described in Figure 1. B, Densitometric analyses are presented as the relative ratio of ICAM-1, VCAM-1, or E-selectin mRNA to cyclophilin mRNA. The relative ratio measured after addition of control buffer is arbitrarily presented as 1. Results were similar in three independent experiments. Bars represent the mean ± S.D. from three experiments. *, p<0.05 versus control buffer. #, p<0.05 versus control buffer plus VEGF (20 ng/ml).

**Figure 5.** Western blot analyses of VCAM-1 and ICAM-1 protein in VEGF-stimulated HUVECs. A, HUVECs were incubated for the indicated times with VEGF<sub>165</sub> (20 ng/ml). Each lane contains 50 µg of cellular protein. B, HUVECs were incubated for 6 h with VEGF<sub>165</sub> (20 ng/ml) in the presence of control buffer (CB) or inhibitors, as described in Figure 2. The western blot was probed with an anti-VCAM-1 antibody or an anti-ICAM-1 antibody (upper panels) and reprobed with an anti-actin antibody (lower panels) to verify equal loading of protein in each lane. Molecular weight markers shown were used to estimate masses (kDa). Results were similar in three independent experiments.

**Figure 6.** Leukocyte adhesiveness in VEGF-stimulated HUVECs. A, Representative phase-contrast photographs of leukocytes adhesion to HUVECs. Note that there are more leukocytes in VEGF-treated HUVECs than in control buffer-treated HUVECs. This effect is changed by co-treatment with various reagents. Bar=50 µm. B, Quantification of the leukocyte adhesion to HUVECs. Leukocytes were labeled with the
Vybrant DiD and added to confluent monolayers of HUVECs which were treated with and without VEGF$_{165}$ (20 ng/ml) for 8 hr and were also treated with control buffer (CB), inhibitors (as described in Figure 2), and anti-ICAM-1 antibody (IA, 10 µg/ml), anti-VCAM-1 antibody (VA, 10 µg/ml), anti-E-selectine antibody (SA, 10 µg/ml), or a triple combination of these antibodies (TA). Then, leukocyte adhesion was measured as described in Materials and Methods. The percentage of leukocytes adhering to HUVECs was calculated by the formula: % adherence = (adherent signal/total signal) x 100. Bars represent the mean ± S.D. from five experiments. *, p<0.05 versus control buffer. #, p<0.05 versus control buffer plus VEGF (20 ng/ml).

**Figure 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), PLC$_{\gamma}$, PI 3' -kinase, and MEK/ERK are activated. NF-$\kappa$B is activated through activation of PLC$_{\gamma}$/SPK/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.
Table 1. Effect of VEGF on production of NO metabolites in HUVECs.

<table>
<thead>
<tr>
<th></th>
<th>Nitrate + Nitrite (nmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Buffer</td>
</tr>
<tr>
<td>Control Buffer</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>L-NAME (3 mM)</td>
<td>0.06 ± 0.02 *</td>
</tr>
<tr>
<td>D-NAME (3 mM)</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Wortmannin (30 nM)</td>
<td>0.08 ± 0.02</td>
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</tbody>
</table>

HUVECs were pretreated with or without NOS and PI 3’-kinase inhibitors for 10 min. Then, VEGF_{165} (20 ng/ml) was added to the cells and incubated for additional 30 min. The NO metabolites in culture media were measured as described in Materials and Methods. Data represent the mean ± S.D. from five experiments. *, p<0.05 versus control buffer. #, p<0.05 versus time VEGF (20 ng/ml) plus control buffer.
Figure 1 (Kim et al.)

A

ICAM-1
VCAM-1
E-selectin
Cyclophilin

<table>
<thead>
<tr>
<th>VEGF (20 ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>0h 1h 2h 4h 6h 8h</td>
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</table>

B

Relative ratio

<table>
<thead>
<tr>
<th>VEGF (20 ng/ml)</th>
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<tbody>
<tr>
<td>0 1 2 4 6 8 (hr)</td>
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Figure 2 (Kim et al.)

A

<table>
<thead>
<tr>
<th>VEGF</th>
<th>CB</th>
<th>SU</th>
<th>CB</th>
<th>SU</th>
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<tbody>
<tr>
<td>ICAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cyclophilin</td>
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</table>

B

**Relative ratio**

<table>
<thead>
<tr>
<th>VEGF (20 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
</tr>
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</table>

* * * * * *
Figure 3 (Kim et al.)

A

<table>
<thead>
<tr>
<th>VEGF</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
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<tr>
<td>4</td>
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<tr>
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<td>O1</td>
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B

<table>
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<tr>
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C

<table>
<thead>
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<th>VEGF</th>
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<tbody>
<tr>
<td>CB</td>
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<td>65</td>
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<tr>
<td>Rb</td>
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<td>cR</td>
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</tr>
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<td>52</td>
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D

Relative ratio

0 0.5 1 2 4 6 (hr)

VEGF (20 ng/ml) VEGF (20 ng/ml)
Figure 4 (Kim et al.)

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>E-selectin</th>
<th>Cyclophilin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L-NAME</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D-NAME</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Insulin</td>
<td>−</td>
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</tr>
<tr>
<td>WT</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

B

Graph showing relative ratio for ICAM-1, VCAM-1, E-selectin, and Cyclophilin.

Legend:
- ICAM-1
- VCAM-1
- E-Selectin

Key:
- *: Significant difference
- #: Highly significant difference

Treatments:
- VEGF
- L-NAME
- D-NAME
- Insulin
- WT
Figure 5 (Kim et al.)

A

<table>
<thead>
<tr>
<th>VEGF (10 ng/ml)</th>
<th>T (hr)</th>
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<tbody>
<tr>
<td>0 2 4 6 8 12 18</td>
<td>6</td>
</tr>
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<table>
<thead>
<tr>
<th></th>
<th>0 2 4 6 8 12 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>220 97.4 66.0 46.0 30.0</td>
</tr>
<tr>
<td>Actin</td>
<td>220 97.4 66.0 46.0 30.0</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>220 97.4 66.0 46.0 30.0</td>
</tr>
<tr>
<td>Actin</td>
<td>220 97.4 66.0 46.0 30.0</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>VEGF (10 ng/ml)</th>
<th>T</th>
</tr>
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<tbody>
<tr>
<td>CB  CB  WT  PD  U7  PT  CC  CB</td>
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<table>
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<td>Actin</td>
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</tr>
<tr>
<td>Actin</td>
<td>220 97.4 66.0 46.0 30.0</td>
</tr>
</tbody>
</table>
Figure 6 (Kim et al.)

A

Control  VEGF  VEGF+SU

VEGF+PT  VEGF+WT  VEGF+TA

B

% Adherence

- Control Buffer
- VEGF

*  # # # # # #
Figure 7 (Kim et al.)

VEGF

VEGFR (FIK-1/KDR) → SU1498

PLCγ → IP3

IP3 → DAG

DAG → Ca2+/eNOS/ Akt

Ca2+ → eNOS

Akt

L-NAME

PLCγ → ?

? → ?

SPK → NO

PKC → NF-κ B

Transcription

ICAM-1

VCAM-1

E-Selectin

PI 3'-Kinase

wortmannin → LY294002

MEK → PD98059

ERK 1/2

U73122

PDTC

Chelerythrine Chloride

Chloride
VEGF stimulates expression of ICAM-1, VCAM-1 and E-selectin through nuclear factor-kappaB activation in endothelial cells
Injune Kim, Sang-Ok Moon, Sung Hoon Kim, Hyung Jin Kim, Young Soon Koh and Gou Young Koh

J. Biol. Chem. published online December 6, 2000

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