Angiopoietin-1 Reduces VEGF-Stimulated Leukocyte Adhesion to Endothelial Cells by Reducing ICAM-1, VCAM-1, and E-Selectin Expression

Injune Kim, Sang-Ok Moon, Sung Kwang Park, Soo Wan Chae, Gou Young Koh

Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) are potent vasculogenic and angiogenic factors that hold promise as a means to produce therapeutic vascularization and angiogenesis. However, VEGF also acts as a proinflammatory cytokine by inducing adhesion molecules that bind leukocytes to endothelial cells, an initial and essential step toward inflammation. In the present study, we used human umbilical vascular endothelial cells (HUVECs) to examine the effect of Ang1 on VEGF-induced expression of three adhesion molecules: intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. Interestingly, Ang1 suppressed VEGF-induced expression of these adhesion molecules. Furthermore, Ang1 reduced VEGF-induced leukocyte adhesion to HUVECs. These results demonstrate that Ang1 counteracts VEGF-induced inflammation by reducing VEGF-induced endothelial adhesiveness.

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Materials and Methods

Two endothelial cell–specific growth factors, vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1), act cooperatively and interactively during prenatal and postnatal vascular development. In fact, co-overexpression of VEGF and Ang1 in mouse skin or rabbit ischemic hindlimb produces an additive increase in vessel formation. Although overexpression of VEGF alone in mouse skin produced profound angiogenesis, it also produced enhanced leukocyte rolling and adhesion, vascular leakage, and inflammation. Thus, VEGF is also a proinflammatory cytokine in addition to being an angiogenic factor. However, co-overexpression of VEGF and Ang1 in mouse skin showed less vascular leakage and inflammation compared with VEGF alone. These data clearly indicate that Ang1 counteracts some subset of activities of VEGF in endothelial cells.

We recently demonstrated that VEGF stimulates the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin mRNAs in endothelial cells through the Flk-1/KDR receptor. This stimulation is mediated through nuclear factor-κB (NF-κB) activation and is suppressed by phosphatidylinositol (PI) 3′-kinase. Other studies have shown that Ang1 strongly activates the PI 3′-kinase/Akt pathway in endothelial cells through its binding to the Tie2 receptor. Therefore, in this study, we assessed the specific role of Ang1 in VEGF-induced expression of adhesion molecules in endothelial cells. Interestingly, our results indicate that Ang1 counteracts VEGF-induced expression and activity of adhesion molecules.

Results and Discussion

We developed a method of RPA by which we can simultaneously detect the mRNA levels of ICAM-1, VCAM-1, E-selectin, and cyclophilin. Because VEGF produces a maximum effect at 4 hours on expression of these adhesion molecules, we examined the effect of VEGF at this time point. VEGF stimulated expression of these adhesion molecules in a dose-dependent manner (Figure 1A). Ang1 (200 ng/mL) inhibited ∼23% to 29%, 46% to 48%, and 62% to 68% of the VEGF-induced ICAM-1, VCAM-1, and E-selectin mRNAs, respectively, at 4 hours and 6 hours, whereas Ang1 (200 ng/mL) by itself did not produce any significant effect on mRNA levels of these adhesion molecules (Figure 1B). A 5-fold molar excess of rTie2-Fc, but not rTie1-Fc, mostly blocked Ang1-induced suppression of VEGF-induced mRNA of adhesion molecules (Figure 1C). These results indicate that Ang1 exerts its effects on endothelial cells through mainly Tie2 receptor binding, but not through Tie1.

We previously demonstrated that VEGF stimulated the expression of ICAM-1, VCAM-1, and E-selectin mRNAs mainly through activation of phospholipase-Cγ and NF-κB. This induction was suppressed by activation of PI 3′-kinase. Because Ang1 is a strong activator of the intracellular PI 3′-kinase/Akt signaling system, Ang1-induced activation of PI 3′-kinase/Akt could be the main pathway for suppressing the VEGF-induced expression of adhesion molecules. As we expected, suppression of basal PI 3′-kinase by the pharmacological inhibitor wortmannin (30 nmol/L) induced expression of adhesion molecules (Figure 1D). Furthermore,
addition of wortmannin (30 nmol/L) not only enhanced VEGF-induced expression of adhesion molecules but also reversed Ang1-induced suppressive effect on VEGF-induced expression of adhesion molecules (Figure 1D). Addition of another PI 3-kinase inhibitor, LY294002 (100 nmol/L), produced the same results (data not shown). In fact, our preliminary results indicated that selective activation (or inactivation) of PI 3-kinase/Akt using adenoviral transfer reduced (or enhanced) VEGF-induced expression of adhesion molecules (data not shown). Thus, Ang1 counteracts VEGF-induced expression of these adhesion molecules, possibly through activation of the PI 3-kinase/Akt pathway.

We looked further at the protein levels of ICAM-1, VCAM-1, and E-selectin in HUVECs treated with Ang1 and VEGF. Consistent with the RPA data, Ang1 (200 ng/mL) by itself did not produce any significant effect, whereas VEGF (20 ng/mL) increased protein levels at 6 hours (Figure 2). Addition of Ang1 (200 ng/mL) inhibited ICAM-1, VCAM-1, and E-selectin protein levels by 43%, 44%, and 62%, respectively. HUVECs were incubated for 4 hours with control buffer (CB), Ang1 (A1, 200 ng/mL), VEGF (VE, 20 ng/mL), or Ang1 plus VEGF (AV). C, HUVECs were incubated for 4 hours with control buffer (CB), Ang1 (A1, 200 ng/mL), VEGF165 (VE, 20 ng/mL), or Ang1 plus VEGF165 (AV) with or without 5-fold molar excess of rTie2-Fc (T2) or rTie1-Fc (T1). D, HUVECs were incubated for 4 hours with control buffer (CB), wortmannin (WT, 30 nmol/L), VEGF165 (VE, 20 ng/mL), wortmannin plus VEGF165 (WV), Ang1 plus VEGF165 (AV), or Ang1 plus VEGF165 plus wortmannin (AVW). Total RNAs (10 μg) were subjected to multiplex RPA probed with 4 antisense RNAs: ICAM-1, VCAM-1, E-selectin, and cyclophilin. Bottom panels, Densitometric analyses are presented as the relative ratio of ICAM-1, VCAM-1, E-selectin, and cyclophilin. The relative ratio measured in the CB is arbitrarily presented as 1. Bars represent the mean±SD from 4 experiments. *P<0.05 vs 0 ng/mL of VEGF165 or CB; **P<0.05 vs VE; and #P<0.05 vs AV.
Ang1 reduces VEGF-induced adhesion molecules

Figure 2. Ang1 suppresses VEGF-induced protein levels of ICAM-1, VCAM-1, and E-selectin in HUVECs. HUVECs were incubated for 6 hours with control buffer (CB), Ang1 (A1, 200 ng/mL), VEGF165 (VE, 20 ng/mL), or Ang1 plus VEGF165 (AV). Each lane contains 50 μg of total protein from the cells. A through C, Western blots were probed with appropriate antibody (top panels). The blots were reprobed with an anti-actin antibody (bottom panels). D, Densitometric analyses are presented as the relative ratio of ICAM-1:actin, VCAM-1:actin, and E-selectin:actin. The relative ratio in the CB is arbitrarily presented as 1. Bars represent the mean±SD from 5 experiments. *P<0.05 vs CB; †P<0.05 vs VE.

Ang1 reduces VEGF-induced adhesion after 8 hours compared with addition of control buffer (Figure 3). Ang1 (200 ng/mL) reduced ~46% of the VEGF-induced leukocyte adhesion (Figure 3). A 5-fold molar excess of rTie2-Fc, but not rTie1-Fc, completely blocked Ang1-induced suppression of VEGF-induced leukocyte adhesion (Figure 3). These results indicate that Ang1 exerts its effects in endothelial cells through Tie2 receptor binding. Function-blocking antibodies to ICAM-1, VCAM-1, and E-selectin, either singly or as a triple combination, suppressed VEGF-induced leukocyte adhesion to varying extents (Figure 3). These data suggest that VEGF-induced adhesiveness requires combined activity of each of these adhesion molecules, because inhibition of the individual molecules could not completely impair the effects of VEGF.

To our knowledge, these results are the first to demonstrate that Ang1 can suppress the expression of adhesion molecules. Furthermore, a recent in vitro experiment demonstrated that Ang1 decreases basal and VEGF-induced endothelial permeability.8 Taken together, Ang1 counters VEGF-induced inflammation in endothelial cells while having an additive effect on vessel formation.2,3 Therefore, combined treatment with VEGF and Ang1 could be better than sole treatment with one for enhancing therapeutic vascularization and angiogenesis while avoiding inflammation.

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References


Key Words: vascular endothelial growth factor ■ angiopoietin ■ adhesion ■ inflammation
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(Ang1 reduces VEGF-induced adhesion molecules)

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Materials and Methods

Materials
We obtained Ang1*, Ang2, soluble Tie1 receptor-Fc (rTie1-Fc) and Tie2 receptor (rTie2-Fc) fusion proteins from Regeneron Pharmaceuticals, Inc. The Ang1* recombinant proteins were produced from CHO cells and purified using affinity chromatography according to the method described by Maisonpierre et al. (1). Ang1* is a recombinant version of Ang1 with modified NH$_2$-terminus and mutated Cys$^{245}$ that is easier to produce and purify. Mutation of Cys$^{245}$ in Ang1, which is not shared between Ang1 and Ang2, does not alter its agonistic properties (1). The purity of the protein was greater than 95% as judged by reducing and non-reducing silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). rTie1-Fc and rTie2-Fc fusion proteins were constructed, produced, and purified according to the method described by Davis et al. (2). Recombinant human vascular endothelial growth factor$_{165}$ (VEGF$_{165}$) was purchased from R&D systems (Minneapolis, MN). PI 3'-kinase inhibitors wortmanin and LY294002 were purchased from RBI, Inc.(Natick, MA). Media and serum were obtained from Life Technology, Inc. (Gaithersburg, MD). 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 (1).

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.

**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.

**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.

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**References**
