Hepatocyte Growth Factor Suppresses Vascular Endothelial Growth Factor–Induced Expression of Endothelial ICAM-1 and VCAM-1 by Inhibiting the Nuclear Factor-κB Pathway

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Abstract—Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are potent angiogenic factors that have been used clinically to induce angiogenesis. However, concerns have been raised about VEGF because of its proinflammatory actions, which include enhancing the adhesion of leukocytes to endothelial cells. We have examined the possible antiinflammatory effects of HGF on the vasculature. HGF, unlike VEGF, did not alter leukocyte adhesion to endothelial cells. Instead it inhibited VEGF-induced leukocyte-endothelial cell interactions and the endothelial expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In a skin inflammation model, VEGF-treated mice showed a significant increase of leukocytes infiltrated or adherent to the luminal surface of blood vessels, as compared with vehicle- or HGF-treated mice. The VEGF effect was markedly suppressed by coadministration of HGF. RT-PCR and promoter analysis revealed that HGF downregulated VEGF-mediated expression of ICAM-1 and VCAM-1 at the transcriptional level. Furthermore, these inhibitory effects coincided with suppression of IκB kinase activity, and this in turn prevented the activation of the inflammatory transcription factor NF-κB. Taken together, our results demonstrate that HGF suppresses VEGF-induced inflammation presumably by inhibiting the endothelial NF-κB pathway. This suggests that combined treatment with HGF and VEGF could be superior to treatment with either factor alone for enhancing therapeutic angiogenesis while avoiding inflammation. **(*Circ Res*. 2005;96:300-307.)**

Key Words: therapeutic angiogenesis ■ inflammation ■ cell adhesion molecule ■ IκB kinase

Vascular endothelial growth factor (VEGF) is endothelial cell–specific because expression of VEGF receptors is essentially restricted to this cell type. It is a potent mediator of angiogenesis in vitro and also leads to functionally significant new vessel formation in several animal models when administered as either the recombinant protein or in the form of DNA encoding VEGF.[1] Preclinical and clinical studies were initially conducted with the aim of using VEGF in conditions involving pathologically decreased blood flow.[2] Subsequently, it was reported to be useful for treating critical limb ischemia, myocardial ischemia, and nonhealing skin ulcers.[3,4] However some recent studies have raised concerns about the harmful effect of VEGF when administrated in vivo[5–8]: it causes enhanced leukocyte rolling and adhesion, vascular leakage, and inflammation.[5,6] It also stimulates the expression of cell adhesion molecules (CAMs) including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin mRNA in endothelial cells, by activating nuclear factor-κB (NF-κB) via the Flk-1/KDR receptor.[9] Thus, VEGF is considered to have a proinflammatory effect on vascular walls in addition to its angiogenic action.

Hepatocyte growth factor (HGF) is also an important angiogenic factor whose activities are well characterized in vitro and in vivo. It is induced in skeletal muscle after ischemic injury[10] and is implicated in capillary endothelial cell (EC) regeneration in the ischemically injured myocardium.[11] HGF has been shown to be effective in causing angiogenesis in several ischemic animal models[12,13] and its clinical utility is currently under investigation. Interestingly, recent studies show that the combination of HGF and VEGF has an additive effect on EC proliferation and a synergistic effect on EC migration in vitro.[14] Moreover, coadministration of HGF and VEGF was more effective in increasing neovas-

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cularization in the rat corneal assay than either growth factor alone.[15] Thus, it appears that combination therapy with HGF and VEGF may be more effective in achieving therapeutic angiogenesis in human coronary disease and peripheral artery disease. Recently, high-density oligonucleotide arrays have been used to identify the molecular basis of the synergistic actions of HGF and VEGF in human endothelial cells.[16] The combination of HGF and VEGF results in the cooperative upregulation of a number of different molecular pathways. However, several genes such as interleukin-6 and interleukin-8 induced by VEGF were reduced by coadministration of HGF.

In this study, we assessed the effect of HGF on VEGF-induced inflammation. HGF itself, unlike VEGF, did not significantly alter leukocyte adhesiveness to endothelial cells. Moreover, it counteracted VEGF-induced leukocyte-EC interactions and the expression of ICAM-1 and VCAM-1 on endothelial cells at the transcriptional level. It also inhibited VEGF-induced activation of the transcription factor NF-κB that is important for the expression of inflammatory genes on the vascular wall. Notably, HGF inhibited VEGF-induced leukocyte adhesion and infiltration in vivo. Overall, our results demonstrate that HGF possesses an antiinflammatory activity counteracting the proinflammatory effect of VEGF in addition to its well-characterized angiogenic effect on the vascular wall. These actions of HGF may enhance its use in treating conditions involving pathologically decreased blood flow.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as described previously[17] and used for experiments in passages 2 to 7. The cells were grown in M199 medium (Invitrogen) supplemented with 20% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 3 ng/mL basic fibroblast growth factor (Upstate Biotechnology), and 5 U/mL heparin at 37°C under a humidified 95% to 5% (vol/vol) mixture of air and CO_2. U937 cells were grown in RPMI-1640 (Invitrogen).

Animals

Pathogen-free female FVB/N mice (8 weeks old) were purchased from Korea Research Institute of Bioscience and Biotechnology (Taejon, South-Korea) and bred at Ewha Womans University (Seoul, South-Korea) for these studies. All experiments were conducted in accordance with the guidelines of the Ewha laboratory animal Genomics Center of the Ewha Womans University, Seoul.

Adhesion Assay

The cells were plated on 2% gelatin–coated 96-well plates at a density of 1×10^4 cells/well and incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) (R&D Systems) or without addition for 8 hours. Then, human U937 cells were added (5×10^4 cells/mL, 200 μL/well) to the confluent monolayers of HUVECs and incubated for 30 minutes. The unattached cells were washed out 3 times with phosphate-buffered saline (PBS), and fixed and stained with Diff-Quick (Baxter Healthcare Corp), and the adherent cells were counted in 5 randomly selected optical fields in each well.

Flow Cytometry Analysis

Cells from subconfluent cultures were detached gently from the wells by treating with PBS containing 2 mmol/L EDTA, washed two or three times with PBS, and resuspended in PBS containing 2% bovine serum albumin. Thereafter, they were incubated with mouse FITC-anti human ICAM-1 and VCAM-1 antibodies (Serotec, Raleigh, NC) for 30 minutes on ice, fixed with 2% paraformaldehyde, and analyzed by flow cytometry in a fluorescence-activated cell sorter (Becton Dickinson).

Semiquantitative RT-PCR Analysis

Total RNA was obtained from the HUVECs with the TRIzol reagent kit (Invitrogen). Different amounts of total RNA (0.5 to 5 μg) were used in the reverse transcriptase-polymerase chain reaction (RT-PCR) assays. cDNA was amplified for 25 cycles with gene-specific primers: 5′-CAGTGACCATCTACAGCTTTCCGG-3′ (sense) and 5′-GCTGCTACCACAGTGATGAT-GACAA-3′ (antisense) for the ICAM-1; 5′-GATACAACCGTCTTGGTCAGCCC-3′ (sense) and 5′-CGCATCCTTCAACTGGCCTT-3′ (antisense) for the VCAM-1. The correlation between the amounts of RNA used and of PCR products obtained with both targets (ICAM-1 and VCAM-1) and with the internal standard (β-actin) was examined.

Reporter Gene Constructs and Luciferase Assays

To construct the ICAM-1 and VCAM-1 luciferase plasmids, we cloned regions spanning −1350 to +45 bp (full length) and −485 to +45 (truncated form) of the human ICAM-1 promoter, and regions spanning −1716 to +119 bp (full length) and −213 to +119 (truncated form) of the human VCAM-1 promoter, into vector pGL3-basic (Promega). HUVECs were transfected with 1 μg of the plasmids and 1 μg of the control pCMV-β-gal plasmid using LipofectAMINE Plus reagents (Invitrogen). Cell extracts were prepared 24 hours after transfection, and luciferase assays were performed using the Luciferase Assay System (Promega). Luciferase activities were normalized with respect to parallel β-galactosidase activities, to correct for differences in transfection efficiency, and the

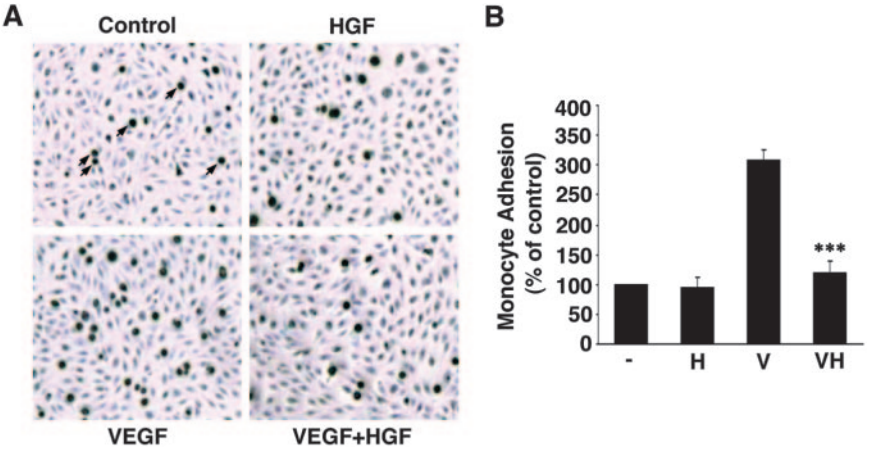

Figure 1. HGF inhibits VEGF-induced leukocyte adhesion to HUVECs. A, HUVECs were incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for 8 hours. Then adhesion to human U937 monocytes was measured as described in Materials and Methods. B, Data are mean±SD relative to adhesion of control untreated cells (set at 100%) in triplicate experiments. H indicates HGF; V, VEGF; VH, VEGF plus HGF. ***$P<0.001$ vs VEGF alone.

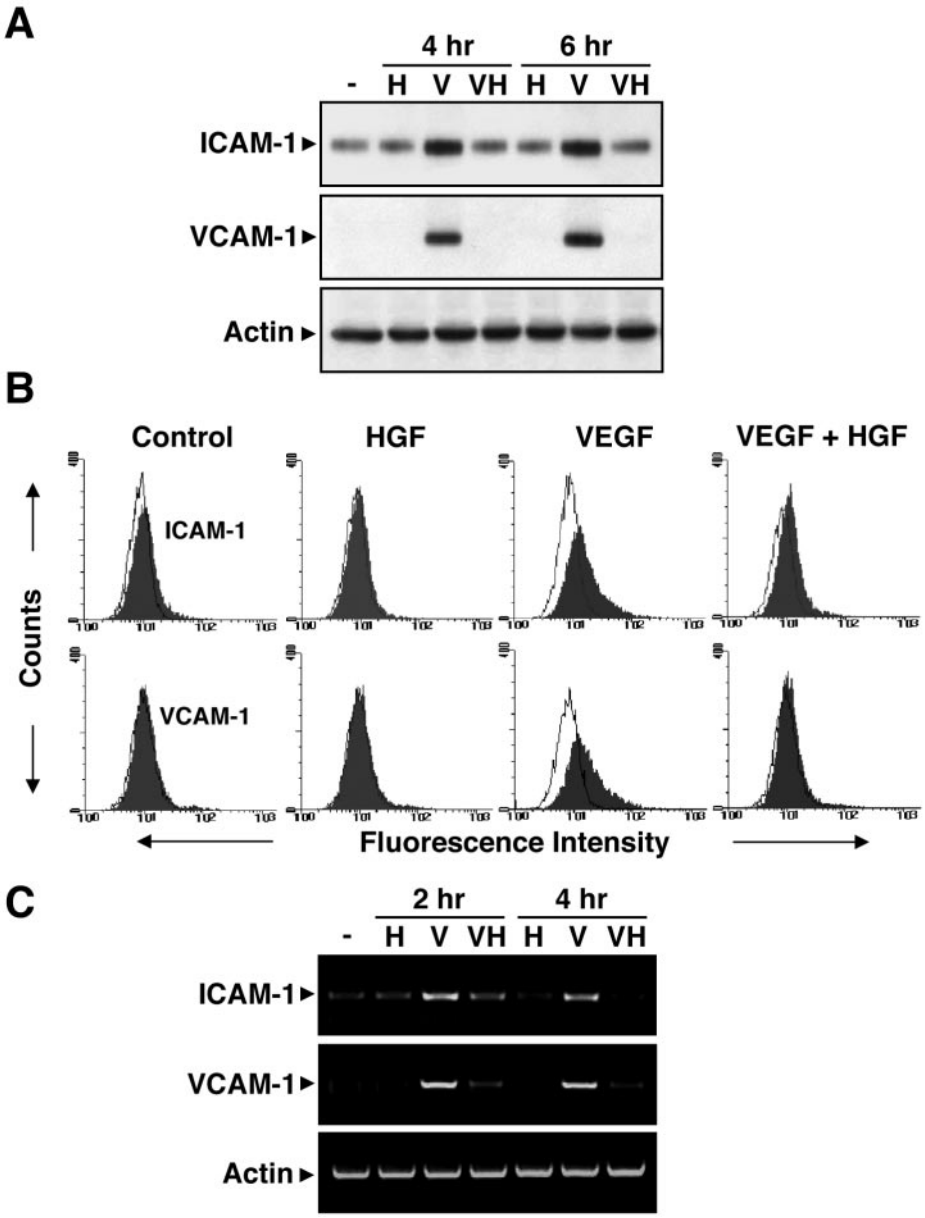

Figure 2. HGF inhibits VEGF-induced expression of ICAM-1 and VCAM-1. HUVECs were incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for the indicated times (A) or 8 hours (B). A, Western blot was probed with anti–ICAM-1 and anti–VCAM-1 antibodies and reprobed with an anti-actin antibody to verify equal loading of protein. B, FACScan analysis with FITC-conjugated ICAM-1 and VCAM-1 antibodies as described in Materials and Methods. C, Total mRNAs were isolated, and semiquantitative RT-PCR was performed using specific primers to human ICAM-1 and VCAM-1. Actin served as an internal control. H indicates HGF; V, VEGF; VH, VEGF plus HGF.

β-galactosidase assays were performed using the β-Galactosidase Enzyme Assay System (Promega).

Electrophoretic Gel Mobility Shift Analysis and In Vitro Kinase Assays

Nuclear protein extracts were prepared from HUVECs as described.[9] NF-κB DNA binding activity was measured in electrophoretic mobility shift assays.[9] Whole tissue extracts were prepared and IKK activity was measured with the immune complex kinase assay[18] after immunoprecipitation with antibody against IKK-γ (Santa Cruz Biotechnology, Santa Cruz, Calif). Recovery of the IKK complexes was assessed by immunoblotting with antibody against IKK-γ.

Immunocytochemical Localization of p65 NF-κB

Nuclear translocation of the p65 subunit of NF-κB was examined by an immunocytochemical method as described previously.[19] Briefly, treated cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. After being washed in PBS, the slides were blocked with 3% bovine serum albumin for 1 hour and the cells incubated with goat polyclonal anti-p65 antibody (Santa Cruz Biotechnology) (1:100). After 2 hours at 4°C, the cells were washed and incubated with anti-goat IgG-rhodamine (Santa Cruz Biotechnology) (1:100) for 1 hour. After staining, the cells were mounted with mounting medium and observed by laser scanning confocal microscopy (Olympus).

Western Blotting

Cell lysates or immunoprecipitates from HUVECs were run in SDS-PAGE and transferred to polyvinylidene diflouride membrane. The blocked membranes were then incubated with the indicated antibodies, and the immunoreactive bands were visualized using a chemiluminescent substrate.

Induction of Delayed-Type Hypersensitivity Reactions

Delayed-type hypersensitivity (DTH) reactions were induced in the skin of mice as previously described.[20] The mice were sensitized by topical application of a 2% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma) solution in acetone/olive oil (4:1 vol/vol) to the shaved abdomen (50 μL) and to each paw (5 μL). Five days after sensitization, mice were anesthetized and received an ear inoculation of VEGF (500 ng), HGF (500 ng), and the mixture of VEGF (500 ng) and HGF (500 ng). Control animals received an injection of vehicle. Four hours after treatment of growth factors, the ears were challenged by topical application of 10 μL of a 1% oxazolone solution.

Histology and Immunohistochemistry

Mice were killed 24 hours after oxazolone challenge. The ears were removed and were embedded in optimal cutting temperature (OCT) compound and frozen. Immunohistochemical stains were performed on 8-μm cryostat sections (5 sections per mouse) using monoclonal rat antibody against mouse CD31 and CD11a (BD Pharmingen).

Perfusion Fixation and Lectin Binding

Twenty-four hours after oxazolone challenge, the retroorbital venous sinus of each animal was injected intravenously with 200 μL biotinylated *Lycopersicin esculentum* lectin (1 mg/mL, Vector Laboratories), which binds to *N*-acetyl-D-glucosamine residues on the luminal surface of vascular endothelial cells.[20] To perfuse the mice, the chest cavity was opened, and the atria were cut to allow outflow of blood and perfusate. Mice were perfused with a fixative (1% paraformaldehyde, 0.5% glutaraldehyde in PBS) via the left ventricle. The ears were removed and the vascular architecture was analyzed in whole mounts of mouse ears, using a ZEISS AxioSkop2 microscope. Images of blood vessels and adherent leukocytes were captured using a ZEISS AxioCam.

Statistical Analysis

The data are presented as mean±SE, and statistical comparisons between groups were performed using 1-way ANOVA followed by the Student *t* test.

Results

HGF Inhibits VEGF-Induced Adhesion of Leukocyte to Endothelial Cells

To determine the effect of HGF on vascular cells in inflammation, we examined whether it increases or reduces leukocyte adhesion to endothelial cells, which is a critical step in vascular inflammation. Confluent monolayers of HUVECs were treated for 12 hours with HGF and the adhesive activity of leukocytes was evaluated. HGF (20 ng/mL) by itself even at high concentrations (up to 100 ng/mL) had no significant effect on leukocyte adhesion to HUVECs (Figure 1). However, 20 ng/mL VEGF produced ≈3-fold increase in leukocyte adhesiveness, and this effect was greatly reduced by cotreatment with HGF (Figure 1).

HGF Suppresses VEGF-Induced ICAM-1 and VCAM-1 Expression in Endothelial Cells

Expression of CAMs, such as ICAM-1 and VCAM-1, on the surface of endothelial cells is required for endothelial-leukocyte interaction. In the absence of inflammation, ICAM-1 and VCAM-1 expression is low on the endothelial cells of most vascular beds, but it is dramatically increased in response to inflammatory cytokines.[21] Therefore, we tested the effect of HGF on ICAM-1 and VCAM-1 expression in endothelial cells. Treatment of HUVECs with HGF alone had no significant effect, whereas VEGF increased expression of ICAM-1 and VCAM-1 in a dose- and time-dependent manner (data not shown), as reported previously.[9] Consistent with its inhibitory effect on VEGF-induced leukocyte adhesion to HUVECs, HGF markedly suppressed the VEGF-induced increase in levels of ICAM-1 and VCAM-1 proteins (Figure 2A). Using flow cytometry, we confirmed that HGF also inhibited VEGF-induced expression of these CAMs on the surface of the HUVECs (Figure 2B).

HGF Inhibits VEGF-Activated mRNA Transcription

To determine whether HGF counteracts VEGF-activated transcription of ICAM-1 and VCAM-1 in endothelial cells,

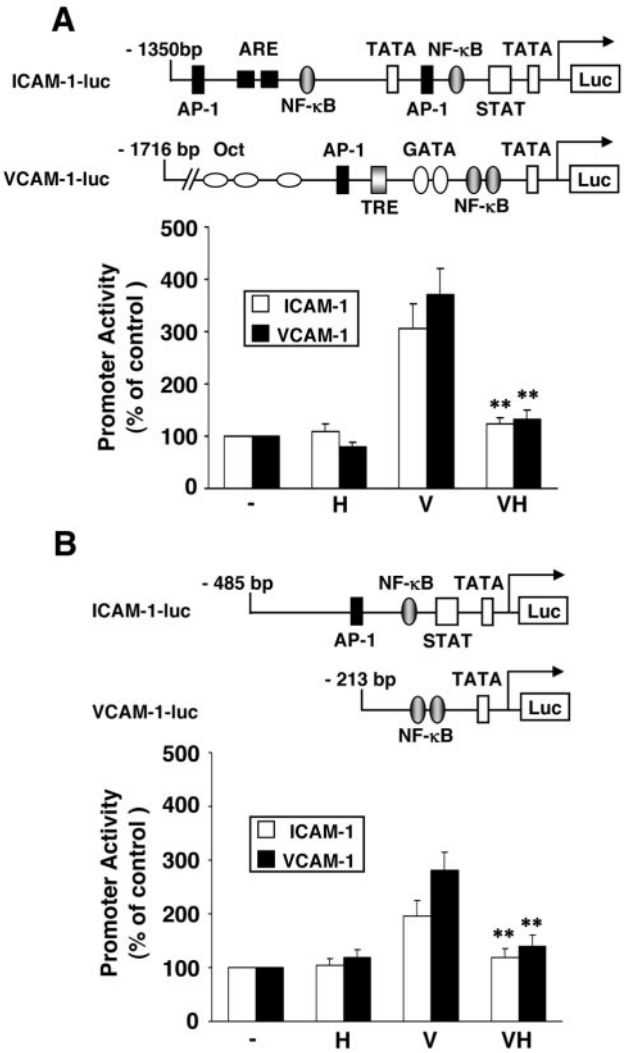

Figure 3. HGF inhibits the promoter activity of the ICAM-1 and VCAM-1 genes. A, Top, Schematic representation of the 5′-promoter regions of the human ICAM-1 and VCAM-1 genes. B, Top, Schematic representation of the 5′–NF-κB motif in the ICAM and VCAM-1 promoters. A and B, HUVECs were transiently transfected with 1 μg of and 1 μg of the control pCMV–β-gal plasmid and incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for 8 hours. Data are mean±SD of triplicate experiments relative to the luciferase light units in control untreated cells (set at 100%). H indicates HGF; V, VEGF; VH, VEGF plus HGF. $^{**}P<0.01$ vs VEGF alone.

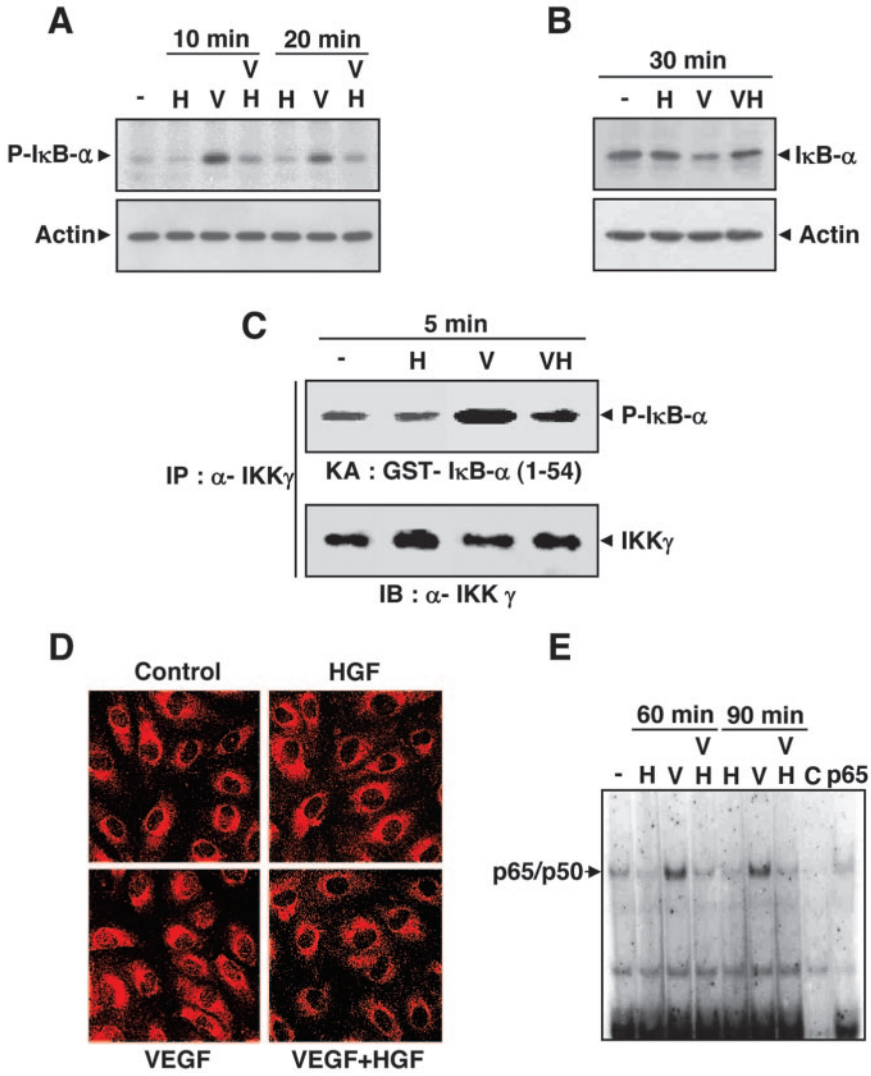

Figure 4. HGF inhibits VEGF-induced NF-κB activation by downregulating IKK activity. A and B, HUVECs were incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for the indicated times. Western blots were probed with anti–phospho-IκB-α (A) and anti–IκB-α antibodies (B). C, IKK activation. IKK complex was immunoprecipitated with anti–IKK-γ antibody (IB) and in vitro kinase assayed with GST-IκB-α fusion protein (KA) and [γ-^{32}P] ATP. D, Immunocytochemical analysis of p65 localization. HUVECs were incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for 30 minutes and subjected to immunocytochemistry as described in Materials and Methods. E, NF-κB activation. Nuclear extracts of the HUVECs incubated with VEGF (20 ng/mL) and/or HGF (20 ng/mL) or without addition, for the indicated times, were incubated in the presence or absence of a 20-fold molar excess of cold human VCAM-1 NF-κB oligonucleotide (lane C) or with antibody specific for p65, before adding radiolabeled oligonucleotide. H indicates HGF; V, VEGF; VH, VEGF plus HGF.

we performed RT-PCR analysis and assays of transcription from the ICAM-1 and VCAM-1 luciferase plasmids described in Materials and Methods. VEGF rapidly induced accumulation of ICAM-1 and VCAM-1 mRNAs and this was almost completely reversed by cotreatment with HGF (Figure 2C). The promoter regions of the ICAM-1 and VCAM-1 genes have been previously described. The human ICAM-1 promoter (1.2 kb) contains binding sites for a number of sequence-specific transcription factors including NF-κB, AP-1, and STAT,[22] and the human VCAM-1 promoter (1.8 kb) includes binding sites for NF-κB, TRE, and GATA.[23] As shown in Figure 3A, activation by VEGF of transcription from the promoter of both ICAM-1 and VCAM-1 was blocked by coadministration of HGF. These results indicate that HGF controls VEGF-mediated expression of ICAM-1 and VCAM-1 at the transcriptional level.

Because the transcription factor NF-κB is involved in VEGF-induced ICAM-1 and VCAM-1 expression in endothelial cells,[9] it seemed possible that HGF suppressed VEGF-induced transcription of these CAMs by inhibiting the NF-κB pathway. Both ICAM-1 and VCAM-1 promoter regions possess proximal NF-κB binding sites located ≈200 bp (ICAM-1) and 65 and 75 bp (VCAM-1) upstream of the transcription start site, respectively.[22,24] To test this possibility, we prepared truncated forms of luciferase plasmids containing the proximal NF-κB binding sites as depicted in the Figure 3B and transfected the resulting plasmids into HUVECs. Consistent with the previous observation, VEGF induced transcription from these truncated ICAM-1 and VCAM-1 promoters (Figure 3B), and this was markedly inhibited by HGF (Figure 3B). Taken together, these results suggest that HGF suppresses VEGF-mediated transcription of ICAM-1 and VCAM-1 by inhibiting the NF-κB pathway.

HGF Inhibits VEGF-Induced NF-κB Activation in Endothelial Cells

The activated form of NF-κB is a heterodimer, which generally consists of a p65 subunit (also called relA) and a p50 subunit.[25] In its inactive state, NF-κB is found in the cytoplasm bound to IκB-α, which prevents it from entering the nucleus.[25] Activation of NF-κB is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκB-α.[26] Therefore, we examined the effect of HGF on VEGF-induced IκB-α phosphorylation and degradation, by

Western blot analysis using antibodies against phosphospecific IκB-α (Ser-32) and IκB-α. Treatment of HUVECs with VEGF led rapidly to phosphorylation of the Ser-32 residue of IκB-α and then to its degradation (Figure 4A and 4B). This process was almost completely reversed by HGF (Figure 4A and 4B). To confirm the inhibitory effect of HGF on VEGF-induced IκB-α phosphorylation, we performed IκB kinase (IKK) assays. IKK is a complex composed of three subunits: IKKα (IKK1), IKKβ (IKK2), and IKKγ (NEMO, IKKAP).[27] IKK activity was immunoprecipitated with anti-IKKγ as described.[28] Cell stimulation with VEGF activated phosphorylation of GST-IκB-α by IKK (Figure 4C), and this effect was inhibited by cotreatment with HGF (Figure 4C).

Release of NF-κB from IκB-α results in translocation of NF-κB to the nucleus, where it binds to specific sequences in the promoter regions of target genes. We next determined the effect of HGF on the nuclear translocation and DNA binding ability of NF-κB. VEGF significantly increased nuclear translocation of the p65 subunit of NF-κB and this effect was abolished by HGF (Figure 4D). Furthermore, VEGF-induced p50-p65 binding to target NF-κB oligonucleotides was also completely blocked by coadministration of HGF (Figure 4E), and HGF on its own had no significant effect on activation of NF-κB. Collectively, these results suggest that HGF suppresses VEGF-induced NF-κB activation by locking the VEGF signaling pathway that leads to IκB-α phosphorylation.

Effect of HGF on TNF-α– or IL-1β–Induced CAM Expression and IκB-α Phosphorylation

The effect of HGF on other inflammatory cytokine-induced CAM expression and NF-κB activation was investigated. HGF by itself did not produce any effect, whereas both TNF-α and IL-1β produced significant increases in ICAM and VCAM-1 expression after 8 hours compared with addition of control buffer (Figure 5A). HGF reduced TNF-α–induced ICAM-1 (≈25%) and VCAM-1 (≈37%) expression as well as IL-1β–induced ICAM-1 (≈34%) and VCAM-1 (≈55%) expression (Figure 5A, 5C, and 5D). In addition, HGF abrogated ≈23% of the TNF-α–induced IκB-α phosphorylation and ≈26% of the IL-1β–induced IκB-α phosphorylation (Figure 5B through 5D). These results suggest that HGF may affect other inflammatory cytokine-induced CAM expression and NF-κB activation in endothelial cells.

HGF Inhibits VEGF-Induced Leukocyte Adhesion and Infiltration In Vivo

To determine the effect of HGF on VEGF-induced inflammation in vivo, we used cutaneous delayed-type hypersensitivity (DTH) reactions, an established experimental model for skin inflammation.[20] The mice were sensitized by topical application of oxazolone for 5 days and received an ear inoculation of vehicle, VEGF, HGF, or the mixture of VEGF and HGF, and then the ears were challenged by topical application of oxazolone for 24 hours. Leukocyte adhesion and infiltration was monitored by in vivo perfusions with the lectin *L esculentum*.[20] VEGF-treated mice showed a significant increase of leukocytes infiltrated or adherent to the luminal surface of blood vessels, as compared with vehicle- or HGF-treated mice (Figure 6A and 6B). This VEGF effect was markedly suppressed by coadministration of HGF (Figure 6A and 6B). Consistently, immunohistochemical analysis revealed that CD11a stains in the ear section were significantly elevated in VEGF-treated mice (by ≈4-fold), as compared with mice cotreated with VEGF and HGF (Figure 6C). However, CD31 stains revealed increased skin vascularization in mice cotreated with HGF and VEGF, as compared with VEGF-treated mice (Figure 6D). With the use of computer-assisted morphometric analysis of CD31-stained sections, we found increased densities of blood vessels in mice treated with HGF (≈50%), VEGF (≈52%), or HGF + VEGF (158%), as compared with vehicle-treated mice (Figure 6E). These results demonstrate that HGF suppresses the inflammatory activity of VEGF in vivo while showing a synergistic effect on angiogenesis when cotreated with VEGF.

Discussion

The present study provides evidence that HGF acts as an antiinflammatory mediator suppressing the proinflammatory action of VEGF in vitro and in vivo. In particular, HGF counteracted the increase in leukocyte adhesiveness to endothelial cells attributable to VEGF that is crucial to the inflammatory reaction. This antiinflammatory effect of HGF is correlated with abrogation of the induction of CAMs in response to VEGF. Previous studies have shown that VEGF upregulates a number of genes including prothrombotic factors, chemokines, and CAMs, many of which depend on the action of NF-κB,[21] which is thought to play a central role in the regulation of inflammatory mediators.[29] Our results show that HGF blocks endothelial NF-κB activation by

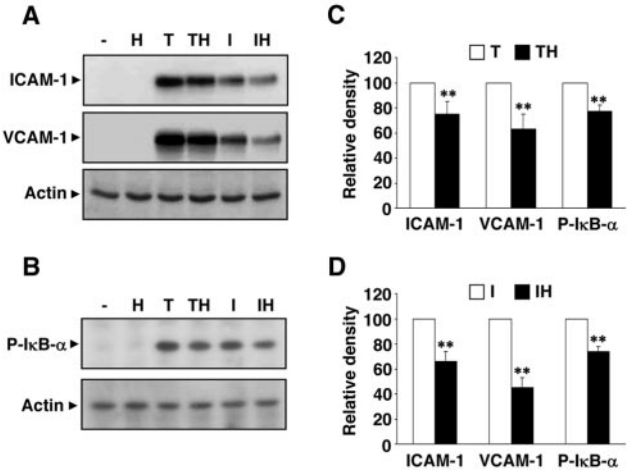

Figure 5. Effect of HGF on TNF-α– or IL-1β–induced CAM expression and IκB-α phosphorylation. HUVECs were incubated with TNF-α (10 ng/mL), IL-1β (50 U/mL), and/or HGF (20 ng/mL) or without addition, for 8 hours (A) or 10 minutes (B). A and B, Western blot was probed with anti–ICAM-1 (A), anti–VCAM-1 (A), and anti–phospho-IκB-α (B) antibodies and reprobed with an anti-actin antibody to verify equal loading of protein. C, Densitometric analyses are presented as the relative ratio of ICAM-1:actin, VCAM-1:actin, and phospho-IκB-α:actin. Relative ratio in the TNF-α or IL-1β alone is arbitrarily presented as 100. Data are expressed as mean±SD from triplicate experiments. H indicates HGF; T, TNF-α;TH, TNF-α plus HGF; I, IL-1β; IH, IL-1β plus HGF. $^{**}P<0.01$ vs TNF-α or IL-1β alone.

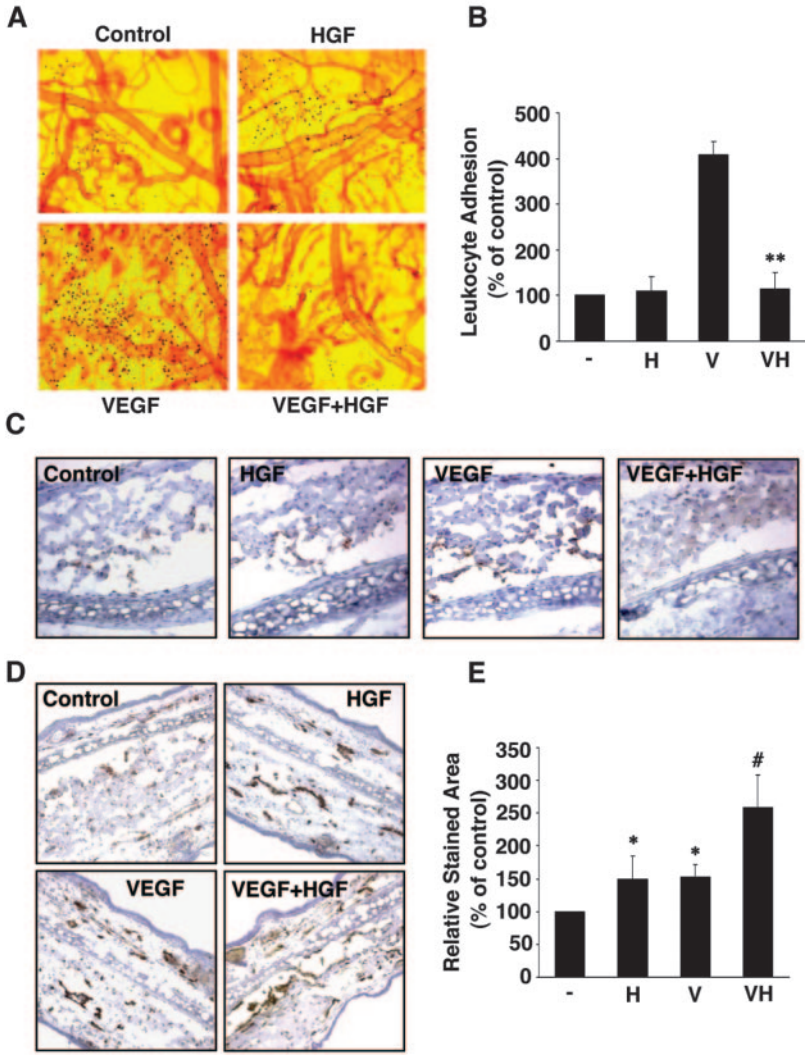

Figure 6. HGF inhibits VEGF-induced leukocyte adhesion and infiltration in vivo. A, Twenty-four hours after oxazolone challenge as described in Materials and Methods, mice (n=5) were perfused with the lectin *L esculentum* to visualize leukocytes infiltrated or adherent to the luminal surface of blood vessels. B, Data are mean±SD relative to the number of infiltrated or adherent leukocytes in the vehicle-treated mice (set at 100%). Leukocytes (C) and endothelial cells (D) in the ear sections were immunostained with antibodies to CD11a and CD31, respectively. E, CD31-stained sections were quantified using of computer-assisted morphometric analysis (Image-Pro Plus software). Data are mean±SD relative to the density of blood vessels in the vehicle-treated mice (set at 100%). H indicates HGF; V, VEGF; VH, VEGF plus HGF. *$P<0.05$ vs control; **$P<0.01$ vs VEGF alone; #$P<0.05$ vs HGF or VEGF alone.

VEGF. This inhibitory action is due, at least in part, to suppression of IKK activity that leads to phosphorylation and subsequent degradation of IκB-α. The inhibitory action of HGF on NF-κB activity is supported by our data showing that HGF prevents the nuclear translocation of NF-κB in response to VEGF (Figure 4). Overall, these data demonstrate that HGF suppresses VEGF-induced inflammation by inhibiting the endothelial NF-κB pathway.

Our data also showed that HGF slightly reduces TNF-α– and IL-1β–induced CAM expression and IκB-α phosphorylation. These HGF effects to TNF-α and IL-1β were relatively weaker than those observed in VEGF. It has been shown that the IKK complex can be activated by various upstream signaling pathways including activation of the mitogen-activated protein kinase kinase kinase (MAP3K) family and IKK recruitment to receptor complexes at the cell membrane.[30] Therefore, our data showing the different inhibitory potency of HGF between VEGF and other cytokines suggest that HGF may affect the specific signaling molecule that is prominently involved in VEGF-stimulated IKK activation. It is noteworthy that Grb2-associated binder-1 (Gab1) directly interacts with mitogen-activated protein/ERK kinase kinase (MEKK)-3 and inhibits MEKK3-dependent NF-κB activation and CAM expression.[31] Considering that Gab1 plays an important role in HGF signaling,[32] the HGF effect may be mediated through Gab1-dependent inhibition of MEKKs and this possibility is under investigation.

Recently, there has been much discussion of therapeutic strategies for inducing stimulating angiogenesis in patients with critical limb ischemia or myocardial infarction. VEGF and HGF are the best documented of the many angiogenic factors in terms of efficacy in preventing the extension of focal ischemic injury. However, the severe side effects of VEGF treatment appear to be important. In mouse skin, overexpression of VEGF produced enhanced leukocyte rolling and adhesion, vascular leakage, and inflammation while promoting profound angiogenesis.[5,6] In addition, administration of VEGF in a rat brain ischemic injury model caused a significant increase in cerebral edema and leakage of the BBB although it was effective in reducing infarct volume.[33,34] However, in contrast to VEGF, transfection of rats with the human HGF gene led to an increase in the area and length of vessels without BBB leakage and exacerbation of cerebral edema.[35] The difference in edema formation between HGF and VEGF is thought to result from a difference in their effects on vascular smooth muscle cells.[35] Our present find-

ings underline the antiinflammatory action of HGF against VEGF-induced inflammation. HGF may therefore be advantageous in therapeutic angiogenesis because of its antiinflammatory action in addition to its angiogenic effect. It is notable that cotreatment of HGF with VEGF synergistically promoted angiogenesis in vitro and in vivo and their underlying angiogenic signaling pathways in endothelial cells[14] (unpublished observation, 2004). Therefore, combined treatment with HGF and VEGF could be better than treatment with either agent on its own in enhancing therapeutic angiogenesis while avoiding inflammation.

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