Monocyte chemoattractant protein-1 (MCP-1) has been recognized as an angiogenic chemokine. In the present study, we investigated the detailed mechanism by which MCP-1 induces angiogenesis. We found that MCP-1 up-regulated hypoxia-inducible factor 1α (HIF-1α) gene expression in human aortic endothelial cells (HAECs), which induced vascular endothelial growth factor-A165 (VEGF-A165) expression in the aortic wall and HAECs through activation of p42/44 mitogen-activated protein kinase (MAPK). In vivo angiogenesis assay using chick chorioallantoic membrane (CAM) showed that MCP-1–induced angiogenesis was as potent as that induced by VEGF-A165 and completely inhibited by a VEGF inhibitor, Flt2. The inhibition of RhoA small G protein did not affect MCP-1–induced VEGF-A165 production and secretion but completely blocked both MCP-1– and VEGF-A–induced new vessel formation, as determined by CAM assay. These results suggest that MCP-1–induced angiogenesis is composed largely of 2 sequential steps: the induction of VEGF-A gene expression by MCP-1 and the subsequent VEGF-A–induced angiogenesis. (Blood. 2005;105:1405-1407)

**Brief report**

**Monocyte chemoattractant protein-1–induced angiogenesis is mediated by vascular endothelial growth factor-A**

Kyung Hee Hong, Jewon Ryu, and Ki Hoon Han

Introduction

Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine family that plays a crucial role in the initiation and progression of inflammation. MCP-1 binds to its specific CC chemokine receptor 2, CCR2, inducing numerous monocyte-mediated proinflammatory signals and monocyte chemotaxis. CCR2 is also expressed in vascular endothelial cells in the arterial wall, and MCP-1–induced activation of CCR2 on these cells is known to be responsible for the regeneration of the endothelial layer after injury and angiogenesis and collateral formation in vivo. These processes may be important in the growth of tumors and inflammatory lesions such as atheromatous plaques; however, the detailed mechanism by which MCP-1 induces angiogenesis is still unclear. The present study demonstrates novel evidences that in vivo MCP-1–induced angiogenesis is mediated through pathways involving vascular endothelial growth factor (VEGF) and the activation of RhoA small G protein (RhoA).

Study design

**Rat aortic ring assay**

Thoracic and abdominal aortas were excised immediately after humane killing from 5-week-old male Sprague-Dawley rats (Samtako, Osan, South Korea). The aorta was transversely cut by 1-mm thickness and embedded in growth factor–deficient Matrigel (Becton Dickinson, Bedford, MA). MCP-1 (10 or 100 ng/mL) was added to the culture media, and microvessel outgrowth was photographed under an IX71 microscope (Olympus, Tokyo, Japan) 48 hours later. The protocol was approved by the Animal Subjects Committee of Asan Medical Center (Seoul, South Korea).

Analysis of HIF-1α protein expression

Cell lysate of cultured human aortic endothelial cells (HAECs; American Type Culture Collection, Manassas, VA) was prepared, and the amount of hypoxia-inducible factor 1α (HIF-1α) protein was estimated by immunoblotting assay as described using mouse monoclonal immunoglobulin G (IgG) detecting HIF-1α (Novus Biologicals, Littleton, CO). The binding of HIF-1α to hypoxia response element (HRE) was analyzed by electrophoretic mobility shift assay (EMSA) as described using [γ-32P]adenosine triphosphate (γ-32P)ATP–end-labeled oligonucleotides containing sequences of HRE 5'-TCTGTACGTGACCACACTCACCTC-3' (Santa Cruz Biotechnology, Santa Cruz, CA).

Analysis of VEGF-A165 mRNA expression

Expression of VEGF-A165 mRNA by excised rat aorta was estimated by real-time polymerase chain reaction (PCR) with SYBR Green I using the specific primers 5'-CCCTGCTTTTACTGTGTAC-3' (sense) and 5'-TCTGAAAAGGCTTCA-CAGTG-3' (antisense). As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified and analyzed under identical conditions using the specific primers 5'-TGGACTCAACGGATTTG-GTCTGTA-3' (sense) and 5'-ATGACTGTGATCGAGTCCCTC-3' (antisense). The Ct value (the cycle number at which emitted fluorescence exceeded an automatically determined threshold) for VEGF-A165 was corrected by the Ct value for GAPDH and expressed as ΔCt. Data were expressed as fold changes of the VEGF mRNA amount, which was calculated as follows: fold changes = 2^ΔCt (ΔCt for control cells – ΔCt for MCP-1–treated cells).

Measurement of VEGF-A165 concentration

A total of 4 × 10^5 HAECs (6 to 8 passages) were cultured on a 24-well plate in endothelial growth medium-2 (EGM-2) complete medium (Clonetics, Walkersville, MD) containing 10% fetal bovine serum (FBS) until 70% to

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measured by ELISA. The results shown are the means ± SE. Mann-Whitney U tests were performed to assess significance of changes compared with controls.

Results and discussion

Our ex vivo rat aortic ring assay showed that 10 ng/mL MCP-1 significantly increased the sprouting of capillaries, whereas a higher MCP-1 concentration (100 ng/mL) was not as effective (Figure 1A). This MCP-1–stimulated capillary formation was completely inhibited by the Rho inhibitor, Clostridium difficile toxin, and by the RhoA inhibitor, C3 transferase. We confirmed CCR2 expression in HAECs and rat arterial wall, as determined by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis. Our preliminary in vitro assays showed that the stimulation of cultured HAEC monolayers with MCP-1 increased HAEC proliferation by 20%, as estimated by the amount of 3H-thymidine incorporated (data not shown). Taken together, our findings strongly suggest that angiogenesis induced by MCP-1 is solely due to the activation of the arterial wall and does not require inflammatory leukocytes.7

The present study demonstrates novel evidences that MCP-1 induces gene expression of HIF-1α by HAECs (Figure 1B). As expected, this increase in HIF-1α expression induced VEGF gene expression. Our RT-PCR and real-time PCR results clearly showed that MCP-1 induced gene expression of VEGF-A165, a main VEGF isoform that induces angiogenesis, by the cultured arterial walls (Figure 1C) and HAECs (data not shown), whereas the mRNA expression levels of VEGF-C and VEGF-D isoforms showed

![Figure 1. MCP-1 up-regulates hypoxia-inducible factor 1α and induces VEGF production by vascular endothelial cells.](image)

![Figure 2. MCP-1–induced angiogenesis in vivo is dependent on VEGF-A and RhoA activation.](image)

Statistical analysis

All data are represented as mean ± SE. Mann-Whitney U tests were performed to assess significance of changes compared with controls.
relatively small changes. A maximal 4-fold increase in VEGF-A165 mRNA expression after MCP-1 stimulation was accompanied by dramatic increases in VEGF-A165 production by HAEC monolayers, as determined by ELISA (Figure 1D). Taken together with previous studies showing VEGF-induced MCP-1 secretion by vascular endothelial cells, positive regulatory feedback loop between VEGF and MCP-1 expression may escalate inflammatory responses and angiogenesis per se. Our data further show the involvement of p42/44 mitogen-activated protein kinase (MAPK) in MCP-1–stimulated VEGF-A production. As previously described, p42/44 MAPK mediates nonhypoxic signals to induce HIF-1α gene expression and enhance HIF-1α stabilization and hence initiates VEGF-induced angiogenesis.

Activation of CCR2, a G protein–coupled receptor (GPCR), triggers numerous signalings, including activation of small G proteins. In our in vivo CAM assays consistently reproduced the results with ex vivo rat aortic ring assays showing that MCP-1–mediated angiogenesis was inhibited by Rho and RhoA inhibitors (Figure 2), suggesting the pivotal role of Rho in MCP-1–induced angiogenesis. However, our ELISA results showed that RhoA inhibition had little effects on MCP-1–stimulated VEGF-A165 production by HAECs (Figure 1C), indicating CCR2–derived RhoA activation is not involved in VEGF regulation. On the other hand, our in vivo CAM assays clearly showed that VEGF-A165–induced angiogenesis was profoundly blocked by RhoA inhibition, indicating RhoA, which is activated by VEGF-A and not by MCP-1, activates endothelial cells to trigger the process of both MCP-1– and VEGF–A165–induced angiogenesis (Figure 2). Previous reports support our findings that Rho is activated by vascular endothelial cells upon stimulation with VEGF. Rho mediates the VEGF–induced migration and proliferation of endothelial cells, and induces tyrosine phosphorylation of several signaling proteins after activation of VEGF receptor 2.

Several reports suggest that other VEGF–independent mechanisms may possibly be involved in MCP-1–induced angiogenesis, such as MCP-1–mediated migration of vascular endothelial cells and Rho–mediated endothelial organization and cytosolic stress fiber formation in the cytosol of HAECs. Our preliminary experiment also confirmed that MCP-1 Rho–dependently induced transmigration and actin polymerization of HAECs (data not shown). However, we clearly observed that MCP-1–induced angiogenesis was completely blocked by a specific VEGF inhibitor, Flt3, indicating that VEGF mainly orchestrates MCP-1–induced angiogenesis (Figure 2).

Taken together, the present study provides compelling evidence that MCP-1–induced angiogenesis is composed largely of 2 sequential steps: the induction of VEGF–A gene expression by MCP-1 and the subsequent VEGF–A–induced angiogenesis. Notably, MCP-1–induced angiogenesis measured as potent as that induced by VEGF–A. Such a potential benefit of MCP-1 on vascular remodeling may provide background for therapeutic use of MCP-1 for arterioocclusive diseases.

References


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