Molecular Mechanism and Role of Endothelial Monocyte Chemoattractant Protein-1 Induction by Vascular Endothelial Growth Factor

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Objective—We investigated the role of monocyte chemoattractant protein-1 (MCP-1) in vascular endothelial growth factor (VEGF)–induced angiogenesis and vascular permeability and the underlying molecular mechanism of VEGF-induced endothelial MCP-1 expression in vitro and in vivo.

Methods and Results—We used an anti–MCP-1 neutralizing antibody for specific inhibition of MCP-1. VEGF increased tubule formation in the angiogenesis assay and vascular permeability in the Miles assay, and these effects were markedly inhibited by anti–MCP-1 antibody. Using a luciferase MCP-1 promoter-gene assay, we found that the activator protein-1 (AP-1) binding site of the MCP-1 promoter region contributes to the increase in MCP-1 promoter activity by VEGF. To specifically inhibit AP-1, we used recombinant adenovirus containing a dominant-negative c-Jun (Ad-DN-c-Jun). Ad-DN-c-Jun inhibited VEGF-induced endothelial MCP-1 mRNA expression and promoter activity in vitro. In vivo gene transfer of DN-c-Jun into rat carotid artery, with the hemagglutinating virus of the Japan liposome method, significantly blocked VEGF-induced MCP-1 and macrophage/monocyte (ED1) expression in endothelium.

Conclusions—These results reveal that endothelial MCP-1 induced by VEGF seems to participate in angiogenesis, vascular leakage, or arteriosclerosis. AP-1 plays a critical role in the molecular mechanism underlying induction of MCP-1 by VEGF. (Arterioscler Thromb Vasc Biol. 2003;23:1996-2001.)

Key Words: angiogenesis ■ vascular permeability ■ gene transfer ■ activator protein-1 ■ dominant-negative mutant

Vascular endothelial growth factor (VEGF) is known as a strong mediator that promotes endothelial cell (EC) proliferation, migration, angiogenesis, and vascular permeability, as reviewed by Carmeliet, and the accumulation of monocytes/macrophages into atherosclerotic lesions in the vessel wall. However, the mechanism of angiogenesis, vascular permeability, and atherosclerosis induced by VEGF is not fully understood. Monocyte chemoattractant protein-1 (MCP-1), 1 of the major chemokines, regulates angiogenesis and vascular remodeling and inflammatory diseases. Although VEGF has been recently reported to increase MCP-1 mRNA expression in cultured ECs in vitro, the pathophysiologic significance of the increase in MCP-1 expression by VEGF is unclear. Furthermore, the molecular mechanism of endothelial MCP-1 induction by VEGF remains to be determined.

In the present study, to examine whether MCP-1 participates in VEGF-mediated angiogenesis and vascular permeability, we used an anti–MCP-1 neutralizing antibody (anti–MCP-1 Ab) to specifically inhibit MCP-1. We also examined the underlying molecular mechanism of VEGF-induced MCP-1 expression in ECs in vitro and in vivo by using a gene-transfer technique. We obtained the first evidence that MCP-1 is an important factor in the process of angiogenesis and vascular leakage induced by VEGF and that activator protein-1 (AP-1) is directly involved in VEGF-induced MCP-1 expression in the vascular endothelium.

Methods

Cell Cultures

Human umbilical aortic endothelial cells (HUAECS) were purchased from Clonetics. Cells were cultured in endothelial basal medium (Clonetics) supplemented with 2% fetal bovines serum (FBS), antibiotics, and growth factors (EGM Single Quots, Clonetics) and used between passages 4 and 6. Bovine aortic endothelial cells (BAECS), purchased from Dainippon Pharmaceutical Co, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplement with 10% FBS, penicillin, and streptomycin.

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Ab) or control mouse IgG1 (R&D Systems) for 11 days; the medium was changed every 3 days; and tubule formation was investigated by staining with an anti-CD31 antibody according to the manufacturer’s instructions. Capillary density was measured with NIH Image software.

Measurement of Angiogenic Activity

An angiogenesis kit (Kurabo Industries), consisting of a 2D coculture system of human umbilical vein endothelial cells (HUVECs)/fibroblasts, was used according to the manufacturer’s instructions.7 To examine the contribution of MCP-1 to VEGF-induced angiogenic activity, recombinant human VEGF165 (10 ng/mL, Oncogene) was added to the basal medium, and cells were incubated in the presence or absence of 1 or 10 μg/mL mouse anti–MCP-1 IgG1 (anti–MCP-1 Ab) or control mouse IgG1 (R&D Systems) for 11 days; the medium was changed every 3 days; and tubule formation was investigated by staining with an anti-CD31 antibody according to the manufacturer’s instructions. Capillary density was measured with NIH Image software.

Miles Assay

This assay has been described by Spyridopoulos et al.9 In brief, 8-week-old male, hairless rats (SLC, Tokyo, Japan), weighing 200 to 250 g, were lightly anesthetized with ether (Sigma), and a 0.5-mL solution of Evan’s blue dye (Sigma) was injected into the tail vein. Twenty minutes later, 0.5 mL VEGF (10 ng/mL), recombinant human MCP-1 (1 ng/mL, Sigma), anti-mouse MCP-1 Ab (1 or 10 μg/mL), or control mouse IgG1 (1 or 10 μg/mL) was intradermally injected. Vascular permeability was assessed by the bleb area colored with blue dye 10 minutes after the injection. The injected area was photographed 10 minutes after injection in all rats, and then no correction for differences in areas of the bleb, each area colored with blue dye was divided by the external area of the bleb.

Adenovirus-Mediated Gene Transfer to ECs

HUAECs and BAECs were infected with a recombinant adenovirus containing a dominant-negative c-Jun (Ad-DN-c-Jun) or control adenovirus at 50 or 100 multiplicity of infection in essential growth medium including growth factors and 2% FBS or in DMEM including 10% FBS, respectively, for 1 hour at 37°C in 5% CO2/95% air; then the medium was replaced with medium 199 containing 0.1% FBS for HUAECs and by DMEM including 0.1% FBS for BAECs, and the cells were incubated for another 24 hours.

Results

Inhibition of MCP-1 Attenuates VEGF-Induced Angiogenic Activity

To estimate the possible role of MCP-1 in VEGF-induced angiogenic activity, we examined the effects of the anti–MCP-1 Ab on VEGF-induced angiogenic activity by testing tubule formation in HUAECs. VEGF increased the capillary density of CD31-positive cells by 1.8-fold over control (P<0.01), and this increase was significantly attenuated nearly to basal levels by 1 or 10 μg/mL anti–MCP-1 Ab (P<0.01) but not significantly by control mouse IgG1 (Figure 1A and 1B).

Inhibition of MCP-1 Attenuates VEGF-Induced Vascular Permeability

To estimate whether MCP-1 is involved in VEGF-induced vascular permeability, a Miles assay was performed. VEGF...
Increased vascular permeability in a dose-dependent manner, and 300 ng/mL VEGF exerted almost a maximal effect (data not shown). VEGF (300 ng/mL) increased vascular permeability by 3.0-fold over control \( (P<0.01) \), which was completely blocked by either 3 or 10 \( \mu \)g/mL anti-MCP-1 (Figure 2A). As shown in Figure 2B, MCP-1 itself increased vascular permeability in a dose-dependent manner, which was significantly attenuated by the anti-MCP-1 Ab (3 \( \mu \)g/mL) to control levels \( (P<0.01) \), whereas the same concentration of control IgG failed to inhibit it (Figure 2B).

**VEGF Increases MCP-1 mRNA Expression and AP-1 Transcriptional Activity in ECs**

VEGF already significantly increased MCP-1 mRNA expression at 3 hours \( (P<0.05) \) in ECs (Figure 3A). Furthermore, to test which transcriptional site of the MCP-1 promoter region participates in VEGF-induced MCP-1 mRNA expression, a dual luciferase assay was performed. Reporter-gene constructs are shown in Figure 3B. In chimeras (P540Luc) containing the entire sequence of the MCP-1 gene promoter, VEGF increased reporter-gene activity by 3.4-fold over control \( (P<0.01) \). In the case of P400Luc, P270Luc, P150Luc, and P540Luc, reporter-gene activity was significantly increased by VEGF \( (P<0.01) \). In contrast, P73Luc, in which 2 TRE sites (AP-1 binding site) were deleted, lost not only its basal activity but also its VEGF-induced promoter activity. To confirm whether the aforementioned TRE sites were indeed involved in VEGF-induced MCP-1 expression, 2 mutants with a specific mutation of each TRE site were used. M1, which has a mutated distal TRE site, retained its inducible activity by VEGF. In contrast, M2, which has a mutated proximal TRE site, lost not only its basal activity but also its VEGF-induced activity (Figure 3C).

**Characteristics of AP-1 DNA Binding Activity Induced by Ad-DN-c-Jun Infection**

VEGF significantly increased AP-1 DNA binding activity in ECs in a time-dependent manner and reached the peak (3.5-fold) at 6 hours (Figure 4A). As shown by the supershift analysis in Figure 4B, the VEGF-induced AP-1 band was supershifted with an anti-c-Fos antibody (sc-253X) or an anti-c-Jun antibody that recognizes the amino-terminal portion (including the transactivation domain) of c-Jun (sc-822X), but not with an anti-c-Jun antibody that recognizes the carboxyl portion of c-Jun (PC06L). On the other hand, Ad-DN-c-Jun infection of ECs generated AP-1 DNA binding activity, whose position was higher than that of the endogenous AP-1 band. The Ad-DN-c-Jun--derived AP-1 band was not supershifted with the anti-c-Fos antibody (sc-253X) or the anti-c-Jun antibody that recognizes the amino-terminal portion (including the transactivation domain) of c-Jun (sc-822X) but was supershifted with the anti-c-Jun antibody that recognizes the carboxyl portion of c-Jun (PC06L, Figure 4B).
Effects of Ad-DN-c-Jun on VEGF-Induced MCP-1 mRNA Expression and AP-1 Transcriptional Activity in ECs

We examined MCP-1 mRNA expression in HUAECs, whereas AP-1 transcriptional activity was assessed in BAECs. Because of unsuccessful transfection of the reporter-gene plasmid in HUAECs, we used BAECs for the reporter-gene assay experiments, as in previous reports.15,16 Interestingly, the time course of MCP-1 mRNA in HUAECs (Figure 3A) after VEGF treatment in our present study was very similar to that in bovine ECs.6 Infection of ECs with Ad-DN-c-Jun at a multiplicity of infection of 50 diminished the increase in VEGF-induced MCP-1 mRNA expression by 44% ($P<0.01$), but infection with the control adenovirus vector did not affect it (Figure 5A). Moreover, as shown by the dual luciferase assay in Figure 5B, VEGF increased AP-1 transcriptional activity in ECs, and this increase was abolished by Ad-DN-c-Jun gene transfer.

Effects of DN-c-Jun on VEGF-Induced MCP-1 Expression In Vivo

To estimate whether c-Jun is involved in VEGF-induced MCP-1 expression in vivo as well as in vitro, we examined the effect of DN-c-Jun gene transfer on VEGF-induced MCP-1 expression in vivo. Figure 6A indicates that the HVJ-liposome complexes with hVEGF165 were successfully transfected into rat carotid artery in vivo, and VEGF was expressed in the endothelial layer of the carotid artery. Furthermore, as shown in Figure 6B, VEGF expression led to significant expression of MCP-1 protein and accumulation of ED1-positive cells in the endothelial layer. However, cotransfection of DN-c-Jun with hVEGF165 prevented the significant expression of MCP-1 protein and the accumulation of ED1-positive cells in the endothelial layer, without effect on VEGF expression. The same experiments were performed in 6 rats per group, and the same results were obtained in all rats. Original magnification ×200.

Discussion

VEGF, a major angiogenic factor, is highly expressed during tumor growth, diabetic retinopathy, and rheumatoid arthritis, as reviewed by Carmeliet,1 and its gene expression is regulated by various stimuli such as hypoxia,17 hyperglycemia,18 and interleukin-1β.19 VEGF induces angiogenesis through VEGF receptors (-1 and -2).20 or the neuropilin 1 receptor as
a coreceptor of VEGF receptor-2.\textsuperscript{21} VEGF also enhances vascular permeability through various intracellular signaling pathways,\textsuperscript{22,23} transendothelial transport named vesicular-vacuolar organelles,\textsuperscript{24} endothelial fenestration,\textsuperscript{25} and nitric oxide–dependent mechanisms.\textsuperscript{26,27} However, the mechanism underlying VEGF-induced angiogenesis and vascular permeability remains to be fully understood. MCP-1, a major chemokine, recruits monocytes, eosinophils, and lymphocytes through its CC chemokine receptor 2, as reviewed in Murdoch and Finn\textsuperscript{5} and Mackay,\textsuperscript{29} thereby contributing to angiogenesis\textsuperscript{3,4,29} and arteriosclerosis.\textsuperscript{30–32} However, it remains to be determined whether or not MCP-1 is involved in VEGF-induced angiogenesis and vascular permeability. These findings, taken together with the recent report that VEGF increases MCP-1 mRNA expression in cultured ECs in vitro,\textsuperscript{6} encouraged us to examine the possible role of MCP-1 in VEGF-induced angiogenesis and vascular permeability by using an anti–MCP-1 Ab.

In the present study, in an in vitro angiogenesis assay, we found that anti–MCP-1 Ab, but not control IgG, significantly blocked VEGF-induced tubule formation (Figure 1). Thus, MCP-1 participates in mediating angiogenesis by VEGF. VEGF is also a potent inducer of vascular leakage. In the present study, of note, the anti–MCP-1 Ab blocked VEGF-induced vascular leakage 10 minutes after injection in the Miles assay (Figure 2A). These observations showed that MCP-1 participates in VEGF-induced acute vascular leakage. Moreover, we found that MCP-1 itself directly enhances vascular leakage in a dose-dependent manner. These results provided the first evidence that endogenous MCP-1 contributes to the acute increase in vascular permeability by VEGF. Thus, MCP-1 seems to participate in mediating angiogenesis and vascular leakage by VEGF.

In the present study, VEGF increased MCP-1 mRNA levels in cultured ECs, in good agreement with a previous report.\textsuperscript{6} However, the molecular mechanism of MCP-1 mRNA induction by VEGF in ECs is unknown. As shown in Figure 3C, our present work demonstrated that the increase in MCP-1 mRNA expression by VEGF was at least in part mediated by the enhancement of MCP-1 promoter activity. However, the increase in MCP-1 mRNA by VEGF is smaller than that in its promoter activity. Thus, it is possible that VEGF might shorten MCP-1 mRNA stability, although further study is needed to elucidate this point. Functional analysis of the MCP-1 promoter and site-specific mutations indicated that the AP-1 binding site (TRE) in the MCP-1 promoter region participates in VEGF-induced MCP-1 promoter activity. These results suggest that the AP-1 binding site might be involved in MCP-1 expression by VEGF in ECs. However, it cannot be completely excluded that MCP-1 expression by VEGF might be partially mediated by an intermediate, because the MCP-1 mRNA increase is prolonged after VEGF treatment.

AP-1 is commonly activated by vascular remodeling–related molecules and plays a central role in the initiation of cellular responses,\textsuperscript{33} including cellular gene expression, growth, migration, or apoptosis.\textsuperscript{34} In this study, VEGF increased AP-1 DNA binding activity in ECs, findings concurrent with a previous work.\textsuperscript{6} Unfortunately, there is no available specific inhibitor of AP-1, which has hampered investigation on the biologic role of AP-1. The proto-oncogene c-Jun is an important component of AP-1. We previously reported that DN-c-Jun, also used in the present work, specifically suppresses AP-1 transcriptional activity\textsuperscript{35} and therefore, is a useful specific inhibitor of AP-1. We also previously demonstrated that DN-c-Jun gene transfer prevents balloon injury–induced intimal hyperplasia\textsuperscript{36} in vivo and platelet-derived growth factor-BB–induced vascular smooth muscle cell proliferation in vitro,\textsuperscript{35} supporting the concept that AP-1 might participate in various vascular diseases. Therefore, to elucidate whether AP-1 contributes to VEGF-induced MCP-1 expression, we performed gene transfer of DN-c-Jun to ECs in vitro and the arterial wall in vivo. As shown by supershift analysis with an anti–c-Fos Ab (sc-253X) and an anti–c-Jun Ab (sc-822X) in Figure 4B, the VEGF-induced endogenous AP-1 in ECs was composed of c-Fos and c-Jun, being similar to our previous reports on AP-1 induced by platelet-derived growth factor-BB in rat smooth muscle cells or human mesangial cells.\textsuperscript{35,37} On the other hand, the AP-1 generated by Ad-DN-c-Jun did not react with the anti–c-Fos Ab (sc-253X) and anti–c-Jun Ab (sc-822X) but did react with the anti–c-Jun Ab (PC06L) that recognizes the carboxyl-terminal portion of c-Jun. These results confirmed the successful expression of DN-c-Jun in ECs. As shown in Figure 5, DN-c-Jun gene transfer attenuated VEGF-induced MCP-1 mRNA expression and MCP-1 promoter gene activity in ECs in vitro. Furthermore, basal MCP-1 promoter activity was also suppressed by DN-c-Jun gene transfer. These observations, taken together with the aforementioned results in the functional analysis of the MCP-1 promoter, show that AP-1 transcriptional activity in ECs in vitro is involved in VEGF-induced MCP-1 induction, but it is possible that its role might not be specific for VEGF.

It is unclear whether or not the important role of AP-1 in VEGF-induced MCP-1 expression in cultured ECs can apply to the in vivo situation. To elucidate this question, we transfected the VEGF gene into rat carotid artery in vivo. As shown in Figure 6A and 6B, the significant amount of VEGF expression by gene transfer led to significant expression of MCP-1 in ECs and the accumulation of ED1-positive cells (monocytes/macrophages) in the endothelial layer. Interestingly, simultaneous gene transfer of DN-c-Jun with VEGF completely abolished the increase in MCP-1 expression or the accumulation of ED1-positive cells. These results show that AP-1 plays a key role in VEGF-induced MCP-1 expression and the subsequent macrophage accumulation in ECs, in vivo as well as in vitro.

In conclusion, this study showed that MCP-1 participates in VEGF-induced angiogenesis and vascular leakage. Furthermore, we obtained the first in vitro and in vivo evidence that specific blockade of AP-1 by a dominant-negative mutant of c-Jun prevents VEGF-induced MCP-1 expression and monocyte/macrophage infiltration into the arterial wall. Thus, AP-1 seems to be the key regulator of VEGF-induced MCP-1 expression. We propose that AP-1 might be a new, useful therapeutic target for various VEGF-related diseases, such as cardiovascular diseases, tumor growth, diabetic retinopathy, or rheumatoid arthritis.
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