p38 Mitogen-Activated Protein Kinase Downregulates Endothelial Progenitor Cells

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Background—Transplantation of endothelial progenitor cells (EPCs) improves neovascularization after ischemia, but patients with coronary artery disease (CAD) or diabetes mellitus show a reduced number of EPCs and impaired functional activity. Therefore, we investigated the effects of risk factors, such as glucose and TNF-α, on the number of EPCs in vitro to elucidate the underlying mechanisms.

Methods and Results—EPCs of patients or healthy subjects were isolated from peripheral blood. Incubation with glucose or TNF-α dose-dependently reduced the number of EPCs (79.9±1.3% and 74.3±8.1% of control; P<0.05, respectively). This reduction was not caused by apoptosis. TNF-α and glucose induced a dose- and time-dependent activation of the p38 MAP kinase, the downstream kinase mitogen- and stress-activated kinase 1, and the transcription factor cAMP-responsive element–binding protein (CREB), in EPCs. Moreover, EPCs from CAD patients had significantly higher basal p38-phosphorylation levels (1.83±0.2-fold increase; P<0.05) compared with healthy subjects. The inhibition of the p38-kinase by SB203580 or infection with a dominant negative p38 kinase adenovirus significantly increased basal number of EPCs (136.7±6.3% and 142.9±18% versus control, respectively). Likewise, ex vivo cultivation of EPCs from patients with CAD with SB203580 significantly increased the number of EPCs and partially reversed the impaired capacity for neovascularization of EPCs in vivo (relative blood flow: 0.40±0.03 versus 0.64±0.08, P<0.05). The increased numbers of EPCs by SB203580 were associated with an augmentation of EPC proliferation and a reduction of cells expressing the monocytic marker proteins CD14 and CD64, suggesting that p38 regulates proliferation and differentiation events.

Conclusions—These results demonstrate that p38 MAP kinase plays a pivotal role in the signal transduction pathways regulating the number of EPCs ex vivo. SB203580 can prevent the negative effects of TNF-α and glucose on the number of EPCs and may be useful to improve the number of EPCs for potential cell therapy. (Circulation. 2005;111:1184-1191.)

Key Words: angiogenesis glucose mitogen-activated protein kinases stem cells tumor necrosis factor-α
variety of cellular stresses, such as TNF-α, in different cell types. It has also been reported previously that high glucose levels can activate the p38 MAPK pathway in many cell types, including endothelial cells. MAPKs are a family of serine/threonine kinases that comprise 3 major subgroups, namely, extracellular signal–regulated kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinases (JNK). Because TNF-α and glucose have been reported to activate the p38, this could be a potential effector signaling mechanism impairing proliferation and differentiation of EPCs. Stimulation of the p38 results in phosphorylation of the downstream kinase mitogen- and stress-activated kinase (MSK) 1 and activation of transcription factors cAMP-responsive element–binding protein (CREB), ATF1, and ATF2, which are involved in the regulation of proliferation and differentiation of hematopoietic progenitor cells and other cell types.

The aim of the present study was to examine the role of MAPK, particularly p38, in the regulation of numbers of EPCs by risk factors for CAD. Our results demonstrate that the reduction of numbers of EPCs induced by TNF-α and glucose in vitro was associated with a profound upregulation of p38 phosphorylation and was completely blocked by p38 inhibitors. Furthermore, EPCs cultivated from patients with CAD showed an increased p38 phosphorylation compared with EPCs from healthy control subjects. TNF-α and high glucose further augmented the phosphorylation of the p38 downstream kinase MSK1 and the transcription factor CREB, whereas ATF2 was not phosphorylated in EPCs, suggesting that MSK1/CREB mediate the p38-dependent effects. Indeed, the inhibitor H89, which inhibits MSK1, also increased numbers of EPCs.

### Methods

#### Study Population and Patient Characteristics

Mononuclear cells were isolated from the peripheral blood of 10 healthy human volunteers and 23 patients with stable CAD documented by angiographic evidence of coronary lesions (Table). Patients with a history of myocardial ischemia documented by the classic symptoms of chest pain, ECG alterations, or elevation of creatine kinase or troponin T within the previous 3 months were excluded. Further exclusion criteria were the presence of active or chronic infection, surgical procedures or trauma within the last 3 months, or evidence for malignant diseases. All women included were in postmenopause and did not take hormone replacement therapy. The Ethics Review Board of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. The mononuclear cells of patients were used to measure number of cells (n=14 patients), phospho-p38 levels (n=10 patients), and relative blood flow (n=5 patients), dependent on the cell number obtained after isolation. The baseline patient characteristics were not different in the subgroups compared with the total patient population.

#### Isolation, Cultivation, and Characterization of EPCs

Mononuclear cells were isolated by density-gradient centrifugation with Biocoll separating solution from the peripheral blood of healthy human volunteers or patients with stable CAD (CAD patients) as described previously. Mononuclear cells (8×10⁶ cells/mL medium) were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; CellSystems) supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamycin, 50 ng/mL amphotericin B, 10 ng/mL epidermal growth factor, and 20% FCS. Mononuclear cells of patients for Dil-Ac-LDL/Lectin staining were suspended in X vivo-15 medium (Biowhittaker) supplemented with 1 ng/mL carrier-free human recombinant vascular endothelial growth factor (VEGF) (R&D), 0.1 μmol/L atorvastatin (provided by Pfizer), and 20% human serum drawn from each individual patient. After 4 days of culture, more than 90% of the EPCs expressed the endothelial marker proteins KDR, VE-cadherin, and von Willebrand factor.

Cells were incubated at the day of isolation (day 0), at day 3 (day 3), or repetitively at days 1, 2, and 3 (days 1 to 3) with TNF-α or glucose without changing the medium.

#### Murine Hindlimb Ischemia Model

The incorporation of EPCs and their contribution to neovascularization were investigated in a murine model of hindlimb ischemia in 8- to 10-week-old athymic NMRI nude mice (The Jackson Laboratory) weighing 18 to 22 g. Ischemia was induced by ligation of the proximal femoral artery, including the superficial and the deep branches, with 7-0 silk suture. All arterial side branches were obliterated by use of an electrical coagulator (Erbe). The overlying skin was closed by use of surgical staples. After 24 hours, mice received an intravenous injection of 5×10⁶ EPCs from healthy volunteers or patients with or without preincubation with the p38 inhibitor SB203580. Two weeks after induction of ischemia, blood flow of the ischemic (right) and normal (left) limb was measured by laser Doppler imager (MoorLDI-Mark 2, Moor Instruments). The perfusion of the ischemic and nonischemic limbs was calculated on the basis of colored histogram pixels (red = high perfusion, blue = low perfusion). Calculated perfusion was expressed as a ratio of right (ischemic) to left (nonischemic) limb.

#### Dil-Ac-LDL/Lectin Staining

Cells were incubated with 2.4 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine–labeled acetylated LDL (Dil-Ac-LDL) (Harbor Bio-Products) for 1 hour. Cells were fixed in 4% paraformaldehyde and counterstained with FITC-labeled lectin from Ulex europaeus (Sigma). Then, 3 to 5 power fields were randomly counted using a computer-based program.

#### Detection of Apoptosis by FACS Analysis

The adherent cells were trypsinized for 2 minutes, then the reaction was stopped with PBS/10% FCS. Then, the cells were washed twice with annexin-binding buffer and incubated with 2.5 μL annexin-PE and 2.5 μL 7AAD for 20 minutes at room temperature according to the instructions of the manufacturer (Pharmingen, Annexin V-PE Apoptosis Kit). Then, cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences).

#### Proliferation Assay

Living cells were incubated with bromodeoxyuridine (BrdU) labeling reagent for 3 days at 37°C. Cells were trypsinized for 2 minutes, the reaction was stopped with PBS/10% FCS, and cells were pelleted.
by centrifugation. Staining was performed according to the manufacturer’s protocol (Roche). Briefly, cells were fixed, digested for 1 hour with DNAse, and then incubated with an anti–BrdU-FITC antibody and thereafter with 1 μg/mL propidium iodide, and analyzed by FACS as previously described.14

Adenoviral Infection
The dominant negative p38 adenovirus was provided by J. Han (The Scripps Research Institute). Antennapedia peptide (Antennapedia internalization sequence RQAIAWPQNRMKWAA) at a concentration of 0.5 mmol/L, adenovirus (20 multiplicity of infection), and 100 μL of OptiMEM (Gibco) were incubated for 30 minutes at room temperature. The mixture was then added to freshly isolated mononuclear cells in 2.5-mL volume and incubated with the cells until day 3.

Western Blot Analysis
Cells were washed and incubated in 75 μL lysis buffer as described previously.13 The nuclear and cytosolic fractions were separated by use of a commercially available kit according to the manufacturer’s protocol (Pierce) as described previously.14 The purity of the nuclear and cytosolic fractions was ensured by immunoblotting with topoisomerase-1.

Proteins (30 to 50 μg/lane) were loaded onto SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Western blots were performed by use of antibodies directed against phospho-p38, phospho-CREB, phospho-ATF2, phospho-ERK1/2, phospho-MSK1, total p38, and total CREB (1:1000; Cell Signaling), β-actin (1:10,000, Sigma), and topoisomerase-1 (1:250; Santa Cruz Biotechnology). Anti-rabbit and anti-mouse secondary antibodies (1:10,000, Sigma) were from Amersham, and anti-goat antibody (1:6000) was from Santa Cruz Biotechnology. Enhanced chemiluminescence was performed according to the instructions of the manufacturer (Amersham). The autoradiographs were scanned and semiquantitatively analyzed, and the protein ratio was calculated (Scion Image).

Protein/DNA–Binding Assay
Protein/DNA arrays were performed according to the manufacturer’s protocol (Panomics). Briefly, 25 μg of nuclear extract was incubated with the TranSignal Probe Mix. DNA/protein complexes were washed. Then, DNA was separated from protein and hybridized on the membranes at 42°C. Signal was detected by use of ECL-Hyperfilm, and semiquantitative analysis was performed by use of Scion Image.

VEGF-ELISA
VEGF levels in the supernatants were measured by a high-sensitivity ELISA (R&D Systems) according to the manufacturer’s protocol. Samples were performed in duplicate.

Flow Cytometry Analysis
Mononuclear cells were incubated with SB203580 (1 μmol/L) after isolation. At day 3, the adherent EPCs were trypsinized and were pooled with the supernatant. A total number of 500,000 cells were incubated in the dark for 15 minutes with an APC-conjugated monoclonal mouse anti-CD14, a PE-conjugated mouse anti-CD64, or a FITC-conjugated anti–von Willebrand factor (BD Biosciences) antibody. Isotype-identical antibodies served as controls. Analysis was performed by use of a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences).

Statistical Analysis
Data are expressed as mean±SEM from at least 3 independent experiments. For in vitro data, statistical analysis was performed by use of a 2-sided Student t test; comparisons between groups were analyzed by t test (2-sided) or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with Bonferroni adjustment. Probability values of P<0.05 were considered statistically significant.

Results
TNF-α and Glucose Reduce EPC Number
Human peripheral blood mononuclear cells were isolated and cultivated as described previously.4 EPCs were characterized as adherent cells after 4 days of cultivation that were double-positive for Di-Ac-LDL uptake and lectin staining. Data are mean±SEM; n=5; *P<0.05 vs control. B, Mononuclear cells were incubated with different concentrations of TNF-α at day 3, and EPCs were counted at day 4. Data are mean±SEM; n=7; *P<0.05 vs control. C, Mononuclear cells were incubated with different concentrations of glucose at day 3, and EPCs were counted at day 4; mannitol served as an osmotic control. Data are mean±SEM; n=5; *P<0.05 vs control. D, Mononuclear cells were grown in EBM with different concentrations of FCS as indicated. At day 3, cells were incubated with TNF-α (10 ng/mL) or glucose (15 mmol/L), and EPCs were counted at day 4. Data are mean±SEM; n=6; *P<0.05 vs control (20%). +P<0.05 vs 2% FCS; §P<0.05 vs 0% FCS.

TNF-α and Glucose Activate p38
The p38-kinase has been shown to be involved in cell differentiation, proliferation, and apoptosis. Therefore, we
investigated whether TNF-α or glucose increases p38 phosphorylation. Incubation of EPCs with TNF-α time- and dose-dependently increased p38-phosphorylation (Figure 2, A and B). The maximum of p38 activity (5-fold phosphorylation) was obtained at 10 minutes of incubation (Figure 2A). Preincubation of the cells for 20 minutes with the specific p38-inhibitor SB203580 (1 μmol/L) prevented p38-phosphorylation by TNF-α (Figure 2A).

Similar results were obtained when EPCs were incubated with high levels of glucose (Figure 2, C and D). Moreover, long-term incubation of EPCs from day 0 with TNF-α and glucose resulted in a significant increase of p38-phosphorylation (Figure 2E). In addition, TNF-α activated the MAP kinase ERK1/2 (Figure 2F). Of note, SB203580 did not inhibit TNF-α-induced ERK1/2 phosphorylation (Figure 2F).

**SB203580 Increases the Number of EPCs**

Having demonstrated that TNF-α and glucose increase p38 and ERK1/2 activity, we next investigated the effect of specific p38 and ERK inhibitors on the number of EPCs. SB203580 significantly increased the number of EPCs under basal conditions and abolished TNF-α- and glucose-induced reduction of the number of EPCs (Figure 3). Likewise, infection of EPCs at day 3 with a dominant negative p38 adenovirus also significantly increased the number of EPCs under basal conditions to an extent similar to that of blockade of p38 with SB203580 (142.9 ± 18% compared with control). In contrast, the inhibitor of ERK activation PD98059 had no effect on the number of EPCs (Figure 3).

**SB203580 Increases the Number of EPCs in Healthy Control Subjects and in Patients With CAD**

To further elucidate a potential clinical relevance, we examined the effect of SB203580 on EPCs of patients with CAD. As reported previously, patients with CAD showed significantly lower levels of ex vivo–cultivated EPCs compared with healthy subjects. Incubation of EPCs with SB203580 at day 3 after isolation increased the number of EPCs of healthy control subjects as well as CAD patients. After incubation...
with SB203580, the number of cultivated EPCs from patients was similar to that of healthy untreated subjects’ EPCs (Figure 4A). Thus, we hypothesized that healthy subjects and CAD patients may have different basal p38 activity. Therefore, we measured the basal p38 activity in healthy subjects as well as in CAD patients. As shown in Figure 4B, p38 activity is increased almost 2-fold under basal conditions in CAD patients compared with healthy subjects.

Because EPCs derived from patients with CAD demonstrated impaired neovascularization in the hindlimb ischemia compared with EPCs from healthy volunteers (Figure 4C), we next investigated whether pretreatment with SB203580 of EPCs derived from patients with CAD would improve recovery of blood flow of ischemic hindlimbs in nude mice. Indeed, infusion of SB203580-pretreated EPCs derived from patients with CAD significantly increased recovery of blood flow in the ischemic hindlimb model compared with untreated EPCs from CAD patients (Figure 4C).

**Effect of p38 on Apoptosis, Proliferation, and Differentiation**

The increased numbers of EPCs after ex vivo incubation of mononuclear cells with the p38 inhibitor could be a result of diverse effects, including the inhibition of apoptosis, stimulation of proliferation, or promotion of differentiation; however, apoptosis, as detected by annexin-FACS staining, was not affected (Figure 5A). In contrast, treatment of EPCs with the p38 inhibitor SB203580 significantly augmented EPC proliferation, as assessed by BrdU staining (Figure 5C).

Next, we assessed the potential influence of p38 inhibition on the release of cytokines such as VEGF, which promotes EPC differentiation and proliferation. However, VEGF levels were similar in SB203580-treated and control cells (Figure 5B), excluding a potential effect of the p38 inhibitor on the expression of this cytokine.

Finally, on the basis of the finding that the p38 kinase enhances the differentiation of macrophages, we investigated whether the p38 inhibitor affects the balance between monocyteic and endothelial cell differentiation. Indeed, coincubation of SB203580 with the total mononuclear cells reduced the relative proportion of cells expressing CD14 and CD64 (Figure 5D). In contrast, p38 inhibition increased the relative proportion of cells expressing the endothelial marker von Willebrand factor (Figure 5E). Thus, these data support the concept that the increase in cell number seen after inhibition of p38 activation depends on increased proliferation as well as on differentiation toward the endothelial cell lineage.

**Downstream Mechanisms**

Next, we determined the downstream targets of p38 mediating the reduction of EPC numbers. After incubation of EPCs and mononuclear cells with TNF-α, p38 is phosphorylated in
the nucleus (Figure 6A). Likewise, the p38-downstream kinase MSK1 and the transcription factor CREB are phosphorylated in a p38-dependent manner in nuclear fractions of EPCs and mononuclear cells after TNF-α/H9251 or glucose treatment (Figure 6, A and B). However, ATF2, another p38-regulated transcription factor, is not phosphorylated by TNF-α/H9251 in EPCs and mononuclear cells (Figure 6A).

To obtain additional data on the transcription factors regulated by p38 inhibition, we used a novel array technology to assess the DNA-binding activity of multiple transcription factors. Incubation of EPCs with glucose significantly increased the DNA-binding activity of CREB in a p38-dependent manner (Figure 6, C and D). In addition, glucose increased the DNA binding of activating protein (AP)-1 and AP-2, whereas a variety of other transcription factors were not affected (Figure 6, C and D).

To finally determine the involvement of the downstream p38 targets involved in activation of CREB, we blocked MSK1. Incubation of EPCs and mononuclear cells with H89, a kinase inhibitor, which, in the concentration used (10 μmol/L), blocks MSK1 and partially blocks protein kinase A, did not affect TNF-α–induced p38 phosphorylation but significantly inhibited MSK1 and CREB phosphorylation (Figure 7A). Moreover, incubation of total mononuclear cells with H89 enhanced the yield of adherent EPCs in the ex vivo culture assay (Figure 7B), suggesting that MSK-1 may be involved in p38-dependent activation of CREB.

**Discussion**

The present study demonstrates that p38 contributes to the reduction of numbers of EPCs induced by TNF-α and high glucose levels in an ex vivo culture assay. Interestingly, although a related MAPK family member, ERK1/2,
activated by TNF-α, its inhibition did not affect the number of EPCs, suggesting that p38 plays a rather specific role in affecting numbers of EPCs. The clinical relevance of these in vitro findings was further supported by the demonstration that EPCs isolated from patients with CAD exhibit an increased p38 phosphorylation. Moreover, the p38 inhibitor SB230580 increased the number of ex vivo–expanded EPCs isolated from patients with CAD. Of note, this effect was detected on top of optimal culture conditions used for clinical stem cell therapy, including statins, which were shown to augment the numbers and functions of EPCs when added to the culture medium. Moreover, pretreatment with SB203580 of EPCs isolated from patients with CAD led to a significant improvement of blood flow in the hindlimb ischemia model, underscoring the in vivo relevance of p38 activity blockade.

p38 activation regulates a variety of downstream signaling cascades. In the present study, we demonstrate that TNF-α and high glucose induced a p38–dependent phosphorylation of MSK1 in the cytosol and in the nucleus. MSK1 is a recently described nuclear CREB and histone H3 kinase that responds to both mitogen- and stress-activated kinases. Indeed, CREB is phosphorylated in EPCs when treated with TNF-α and high glucose. MSK1 and subsequently CREB can be activated by the p38 kinase and by ERK1/2. Here, we show that the p38 kinase inhibition and MSK1 inhibition, but not ERK1/2 inhibition, had an effect on the number of EPCs, suggesting that the effects mediated by TNF-α and high-glucose treatment are independent of ERK1/2. This is in line with findings by Han et al., who demonstrated that p38 kinase plays the predominant role in the activation of MSK1 and CREB but not ERK1/2.

In contrast to CREB phosphorylation, the phosphorylation of ATF2 was unchanged on p38 inhibition or activation in EPCs, suggesting that ATF2 is not a target for p38 kinase under these experimental conditions. Similar findings were reported by Fuchs et al., who demonstrated that the activation of ATF2 in response to cellular stress requires the stress-activated protein kinase but is independent of p38.

The p38 and its downstream targets critically regulate a variety of biological responses, including apoptosis, proliferation, and differentiation. The data of the present study demonstrate that p38 does not directly affect EPC apoptosis and does not interfere with the release of VEGF. In contrast, the proliferation of EPCs was significantly augmented after treatment of EPCs with the p38 inhibitor SB230580. p38 is known to regulate proliferation of myeloid and erythroid progenitor cells. The increased proliferation might be well explained by p38-dependent phosphorylation and inhibition of CREB. CREB is known to bind to the promoter region of cyclin D1 and thereby inhibits proliferation. Accordingly, we demonstrated that long-term incubation with SB203580 led to a significant increase in cyclin D1 expression (data not shown). Moreover, we identified 2 other transcription factors, namely, AP-1 and AP-2, which were regulated in a manner similar to CREB. Glucose-induced p38 activation has been shown to activate AP-1 in endothelial cells and regulate inflammatory cytokines. However, the biological function of AP-1 in EPCs is not yet clear. Therefore, further studies are necessary to elucidate the biological importance of AP-1 and AP-2 activation in EPCs.

p38 is a critical regulator of cell differentiation. In particular, p38-dependent phosphorylation of CREB was shown to mediate the differentiation of macrophages induced by granulocyte colony–stimulating factor. Interestingly, the p38 inhibitor modulated the ratio of endothelial and monocyte committed cells in the culture assay in favor of the endothelial lineage. Whereas the yield of EPCs out of total peripheral blood mononuclear cells was significantly augmented, monocytes, which are characterized by expression of CD14 and CD64, were significantly reduced. Therefore, one may speculate that p38 inhibition prevents progenitor cell differentiation toward the macrophage lineage and thereby facilitates endothelial cell differentiation. p38 inhibitors have previously been shown to increase VEGF-induced angiogenesis. Interestingly, it was demonstrated that concomitantly, the p38 inhibitor blocked hyperpermeability induced by VEGF. The combination of improved angiogenesis and EPC levels but, on the other hand, reduced hyperpermeability and inflammation might be an attractive profile for a potential proangiogenic therapy.

Taken together, the results of the present study demonstrate a critical role of p38 and its direct downstream kinase MSK1 and CREB for the regulation of numbers of EPCs. Inhibition of p38 increases proliferation and enhances endothelial differentiation ex vivo. Therefore, p38 inhibitors might be promising tools to augment the yield of ex vivo–expanded EPCs for cell therapy.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (FOR501, Di600/6-1). We thank Tina Rasper, Christine Goy, and Sarah Jainski for expert technical assistance.

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Circulation. 2005;111:1184-1191
doi: 10.1161/01.CIR.0000157156.85397.A1
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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