Objective—Function and availability of circulating progenitor cells (CPC) have been shown to be impaired in patients with diabetes mellitus (DM). Therefore, the aim of the present study was to analyze possible mechanisms leading to the reduction of CPC amount and function.

Methods and Results—Peripheral blood mononuclear cells (MNCs) of healthy donors (n=15) were cultivated under hyperglycemia (HG) conditions (12 mmol/L D-Glucose) or in osmotic control medium (Con) (5 mmol/L D-Glucose plus 7 mmol/L L-Glucose) for 7 days. CPC amount was determined by uptake of acetylated low-density lipoprotein and lectin binding. On the functional level, cell cycle status, nitric oxide (NO) production, and migrational and integrative capacity of CPCs were assessed. HG conditions caused a significant decrease in CPC amount derived from healthy MNCs. Furthermore, HG conditions led to a functional impairment, reflected in a decreased NO production and matrix metalloproteinase (MMP)-9 activity, as well as an impairment of the migrational and integrative capacities.

Conclusion—HG, a main feature of DM, affects important functional characteristics of CPCs. These results may provide further insight into the pathomechanism of endothelial dysfunction in HG. (Arterioscler Thromb Vasc Biol. 2005; 25:698-703.)

Key Words: circulating progenitor cells ■ endothelium ■ hyperglycemia ■ nitric oxide ■ vascular biology

Diabetes mellitus has become the most important single cause for vascular diseases in the industrialized world. Atherosclerosis and endothelial dysfunction develop as elevated levels of reactive oxygen intermediates and advanced glycosylated end products injure the endothelial cell layer. Because the onset and progression of complications are delayed in diabetic patients with good glycemic control, hyperglycemia is thought to be a key factor in the development of endothelial dysfunction and atherosclerotic lesions.

During the past years, circulating progenitor cells (CPCs) derived from the bone marrow have been shown to contribute to the preservation of an intact endothelial layer and to the revascularization of ischemic tissue. Recently, 2 theories have evolved discussing the participation of progenitor cells with endothelial lineage commitment to endothelial repair and revascularization: CPCs are thought to contribute to the process of vasculogenesis, which comprises the adhesion of CPCs to sites of vascularization and their subsequent infiltration and partial digestion of the target tissue resulting in the growth of a new blood vessel; and the adhesion of CPCs to activated endothelium is believed to reconstitute dysfunctional endothelium, caused by secretion of mediators of proliferation and migration, as well as survival factors. Thus, CPC function depends on several steps and its regulation is highly complex. Recently, endothelial nitric oxide synthase (eNOS) expression and phosphorylation was identified to be essential not only for survival, migration, and angiogenic response of mature endothelial cells but also for CPCs. Besides its vasodilatory effects, eNOS-derived nitric oxide (NO) has been shown to promote the release of CPC from the bone marrow through nitrosylation and activation of matrix metalloproteinase (MMP)-9 and an increased vascular endothelial growth factor (VEGF) expression. VEGF is known to feed-back on protein kinase B (AKT) activity and eNOS phosphorylation at the serine residue 1177 (Ser1177) and, hence, is able to increase the number of CPCs through AKT-mediated eNOS-dependent and eNOS-independent effects. The AKT counterplayer protein phosphatase 2A (PP2A) dephosphorylates eNOS at Ser1177, a process which is associated with the decline in NO production. CPC count and function are altered in patients with cardiovascular risk factors, especially in those with diabetes mellitus, but the underlying mechanisms are not fully understood yet.

Therefore, it was the aim of the present study to determine whether hyperglycemia affects CPC survival in the cell culture because of an induction of apoptosis and an inhibition of proliferation on the basis of a cell cycle arrest. Moreover, we sought to determine whether CPC function is reduced in
hyperglycemic conditions because of changes in the activity of MMPs and alterations of eNOS-mediated NO production.

Methods

Study Population
We recruited healthy adults between ages 18 and 45 years. Individuals with cardiovascular risk factors and pregnancy or a known history of tumor disease, which might influence vascularization, were excluded. The study protocol was approved by the local ethics committee of the University of Leipzig.

Cell Culture
Mononuclear cells were isolated from peripheral blood and cultured as previously described.17 For more detailed information, please see http://atvb.ahajournals.org.

Characterization of Adhering Cells and Determination of CPC Number
The adhering cell population was characterized by flow cytometry for the expression of VE-cadherin, CD3, CD133 (BD Pharmingen, San Jose, Calif), CD34 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), VEG-receptor 2 (KDR; R&D Systems, Wiesbaden, Germany), CD11b, CD14, and CD68 (DAKO, Glostrup, Denmark). For more detailed information, please see http://atvb.ahajournals.org.

MMP-9 inhibition experiments, the MMP-9 Inhibitor I (Calbiochem, San Jose, Calif), CD34 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), CD11b, CD14, and CD68 (DAKO, Glostrup, Denmark). The amount of CPC was assessed by binding of 1,1-dioctadecyl-3,3',3'-tetramethylindocarbocyanine–labeled acetylated low-density lipoproteins (Dil–acLDL; Molecular Probes, Leiden, the Netherlands) and uptake of fluorescein isothiocyanate (FITC)-labeled Ulex europaeus lectin (FITC-lectin; Sigma).

eNOS Activity and Phosphorylation
The eNOS phosphorylation at Ser1177 was investigated by Western blot analysis and normalized to GAPDH expression as previously described.18 NO release from CPCs was measured as the NO–nitrite accumulation from CPCs grown on cover slides. The proliferation of CPCs was assessed by bromodeoxyuridine (BrdU) incorporation.

Matrix Metalloproteinase 9 Expression and Activity
mRNA expression of MMP-9 and TIMP1 was analyzed by real-time reverse-transcription polymerase chain reaction and normalized to the amount of 18s rRNA in the same sample. The variability of the mRNA expression analyses was <1% for duplicate measurements. The activity of MMP-9 was assessed by gelatin zymography. For MMP-9 inhibition experiments, the MMP-9 Inhibitor I (Calbiochem, Darmstadt, Germany) was applied at a concentration of 50 nM.

Apoptotic Rate
Apoptosis was analyzed in the adherent cell fraction by caspase 3 activity assay (Roche, Heidelberg, Germany) and by measuring the enrichment of free histone protein (Cell Death Detection ELISA; Roche).

Cell Cycle and Proliferation Analysis
Cell cycle analysis was performed using propidium iodide staining.20 mRNA expression of p16Ink-4a and p21Waf-1 was determined in adherent cells by real-time reverse-transcription polymerase chain reaction and normalized to 18s rRNA.

Results

Subject Characteristics
Fifteen healthy adults, 12 men and 3 women, with an average age of 31.6±1.8 years, were included in this study. None of the participants was adipose (body mass index, 23.0±0.66 kg/m²) or displayed elevated glucose levels (glucose level, 4.4±0.2 mmol/L; HbA1c, 5.1±0.1%).

Fluorescence-Activated Cell Sorter Characterization of Adhering Cells
Progenitor cells in the adhering cell fraction were characterized flow cytometrically. A proportion of the adhering cell population displayed endothelial (KDR, 61.8±5.9%; VE-cadherin, 60.4±7.3%), as well as progenitor-like antigen expression (CD133, 15.8±3.2%; CD34, 33.4±5.6%). A minority of adhering cells displayed leukocyte or monocyte/macrophage typical cell surface markers (CD3, 6.5±1.8%; CD11b, 10.9±1.8%; CD14, 8.7±2.3%; or CD68, 5.3±1.2%).

eNOS Phosphorylation and NO Production
Culture of CPCs in hyperglycemic medium led to a decrease in eNOS phosphorylation at Ser1177 by 87% as compared with osmotic control conditions (HG, 0.24±0.13 versus Con, 1.89±0.53 arbitrary units; P=0.03) (Figure 1A). This reduc-
network-like structures of mature human coronary artery endothelial cells was diminished by 50% as compared with CPCs cultured in control medium (HG, 14.9 ± 1.8 versus Con, 27.1 ± 3.6 CPCs per 100 endothelial cells (ECs) in a structure; P = 0.005). This effect was partly blunted after incubation with OA (HG +OA, 20.5 ± 2.2 CPCs per 100 ECs in a structure; P = 0.04 versus HG) (Figure 2B).

**MMP Expression and Activity**

MMP-9 mRNA expression was significantly downregulated (HG, 1.2 ± 0.4 versus Con, 1.9 ± 0.5 arbitrary units; P = 0.04), whereas the MMP-9 inhibitor TIMP1 was upregulated in cells cultured under hyperglycemic conditions (HG, 1.4 ± 0.3 versus Con, 1.0 ± 0.2; P = 0.02). Consistently, gelatin zymography revealed a reduction in MMP-9 activity by 44% in CPCs cultured in hyperglycemic medium as compared with CPCs grown in osmotic control medium (HG, 2.21 ± 0.54 versus Con, 4.98 ± 0.58 arbitrary units; P = 0.04). MMP-9 activity was elevated again by PP2A inhibition in the HG group (HG +OA, 3.11 ± 0.53 arbitrary units; P = not significant) (Figure 2C). Moreover, MMP-9 activity was directly related to the invasive/integrative capacity of CPCs assessed by matrigel assay (r = 0.54; P = 0.002) (Figure 2D). MMP-9 inhibition (MI) led to a decrease in MMP-9 activity (Con + MI, 3.28 ± 0.34; P = 0.02 versus Con; HG + MI, 1.59 ± 0.46; P = 0.01 versus HG) and invasive activity (Con + MI, 10.5 ± 5.0; P = 0.04 versus Con; HG + MI, 8.4 ± 3.9; P = 0.1 versus HG). The involvement of eNOS-mediated NO production in the regulation of MMP-9 activity and invasive activity of CPCs was analyzed after overnight preincubation with l-NMMA. eNOS inhibition resulted in a decrease of MMP-9 activity in the control group but did not further deteriorate the reduction of MMP-9 activity in the HG group (Con + l-NMMA, 3.78 ± 1.31; P = 0.01 versus Con; HG + l-NMMA, 2.85 ± 0.91; P = 0.4 versus HG). Consistently, invasive activity of CPCs was found to be decreased in the presence of l-NMMA in the control group, but not in the HG group (Con + l-NMMA, 17.1 ± 2.1; P = 0.04 versus Con; HG + l-NMMA, 15.6 ± 3.0; P = 0.9 versus HG).

**Impact of Hyperglycemia on the Amount of CPC**

The number of CPCs as assessed by DiL–acLDL and FITC lectin staining was decreased by 33% after 7-day culture in hyperglycemic medium compared with osmotic control medium (HG, 18.1 ± 6.5 versus Con, 27.4 ± 10.0 CPCs per 1 × 10^5 plated mononuclear cells; P = 0.04) (Figure 3A).
Effect of Hyperglycemia on Apoptosis

Exposure of CPCs to hyperglycemia caused a 50% increase in the rate of apoptotic cell death (HG, 0.18±0.06 versus Con, 0.12±0.04 arbitrary units; P=0.04) as assessed by the abundance of free histone proteins (Figure 3C). Concomitantly, caspase 3 activity was increased by 30% in the HG group (HG, 56.8±7.8 versus Con, 41.5±4.7 arbitrary units; P=0.01).

Analysis of Cell Proliferation and Cell Cycle

**Proliferation Rate**

BrdU incorporation was measured to assess the number of proliferating CPCs in the cell culture. Proliferation of CPC grown in HG was reduced by 50% as compared with the Con group (P=0.01) (Figure 3B).

**Cell-Cycle Analysis**

Propidium iodide staining of the adherent cell population revealed that after long-term exposure to hyperglycemia, less CPCs were found in the S phase (HG, 0.83±0.16 versus Con, 1.95±0.18%; P=0.04) and G2/M phase (HG, 0.08±0.01 versus Con, 0.37±0.03%; P=0.01) of the cell cycle compared with CPCs grown in osmotic control medium, which is consistent with the proliferation data.

**p16Ink-4a and p21Waf-1 Expression**

The mRNA expression of the cell cycle inhibitors p16Ink-4a (HG, 0.81±0.25 versus Con, 0.60±0.23 arbitrary units; P=0.047) and p21Waf-1 (HG, 1.37±0.32 versus Con, 0.73±0.15 arbitrary units; P=0.02) was significantly increased by 35% and 88%, respectively, after incubation in HG medium. Concomitantly, the amount of p16Ink-4a (HG, 10.1±3.7 versus Con, 6.4±1.9% of total CPC count; P=0.04) and p21Waf-1 (HG, 59.7±7.4 versus Con, 36.1±2.3% of total CPC count; P=0.02) expressing CPCs was significantly elevated after exposure to hyperglycemic medium as compared with control medium. Staining of adhering CPCs confirmed nuclear localization of the antibody target (Figure 1, available online at http://atvb.ahajournals.org).

Discussion

Hyperglycemia contributes to endothelial dysfunction and plays a pivotal role for the development of vascular complications in patients with diabetes mellitus.4,21,22 This has been partially attributed to a reduction in CPC count and an impairment of CPC function.14–16 but the underlying mechanisms are not fully understood yet.

In this cell culture study, we used hyperglycemia as an in vitro model of diabetes mellitus and hypothesized that hyperglycemia is an important factor mediating the described decline in CPC count and function in diabetes mellitus. Here, we report that an increase in apoptosis and a decrease in CPC proliferation participate in hyperglycemia-induced reduction in CPC count. Moreover, on the functional level, hyperglycemia caused a reduction in MMP-9 activity that is associated with a decreased capability of CPCs to invade a target tissue and incorporate into tubular structures. Furthermore, hyperglycemia was identified to enhance PP2A activity in CPCs, which resulted in a declined eNOS phosphorylation at Ser1177 and, consequently, NO liberation. Altogether, these findings provide mechanisms possibly contributing to the impairment of CPC count and function in patients with diabetes.

In our study, we addressed the effect of HG on nonterminally differentiated circulating progenitor cells. However, we did not enrich for CD34-positive cells before culture for several reasons: commercially available selection methods like fluorescence-activated cell sorter sorting or positive selection using magnetic beads are likely to influence cell signaling and sometimes sorted cells are more prone to cell death. Additionally, these methods do not achieve 100% purity, leaving a certain amount of undesired cells. Furthermore, survival and differentiation of CPCs depends on interaction with other cell types, which influence signaling through direct cell–cell contact or secretion of growth, survival, and differentiation factors, of which several are unknown, so far.

In comparison to most cell culture studies, we chose a lower glucose concentration (12 mmol/L) to simulate hyperglycemia, because it resembles more the conditions in diabetic patients. L-glucose was chosen as an osmolar control because it is known to have no direct impact on glucose metabolism. Initially, mannitol as a second osmolar control was applied, but because no significant differences were observed between L-glucose and mannitol, the mannitol control was omitted. Furthermore, in vitro studies on hyperglycemia assessing various characteristics of different cell types found effects in the hyperglycemia group, whereas no effects were described in both osmotic control groups, neither L-glucose–supplemented nor mannitol-supplemented.23,24

Impact of Hyperglycemia on CPC Amount

As demonstrated in this report, culture of mononuclear cells in hyperglycemic medium resulted in a significant reduction in CPC count compared with osmotic control conditions. This in vitro finding is in accordance with in vivo results published by other groups that reported a diminished number of CPCs in patients with diabetes mellitus.14–16 In addition, Loomans et al reported an inverse correlation between CPC number and HbA1C levels in diabetic patients.15

In patients with type I diabetes, 2 different mechanisms are potentially contributing to the reduction in CPC number: limited gain in cell number (impaired proliferation) and/or augmented cell loss (necrosis/apoptosis).

In diabetic patients and in cell culture models, cell-cycle progression and proliferation of mature ECs have been found to be affected.25–27 Our data, furthermore, show an accumulation of CPCs in the G2/M phase, and a diminished number of CPCs was found in the G0/G1 and S phases after exposure to hyperglycemic medium. These findings are consistent with the upregulation of p16Ink-4a and p21Waf-1, involved in the regulation of G1-S–phase transition and the finding of a decreased number of proliferating CPCs in hyperglycemia. Thus, our in vitro data might explain why the CPCs from patients with diabetes mellitus display reduced proliferative capacity as compared with those of healthy controls.15 Altered expression levels of p16Ink-4a and p21Waf-1 are also associated with apoptosis, as reported for several cell types.28–30 Consistent with these data, in our study the
HG-induced upregulation of p16\textsuperscript{13–14} and p21\textsuperscript{9,10–11} is also accompanied by an increase in apoptosis rate.

From these findings, it seems that loss of surviving CPCs in HG is a multifactorial phenomenon, resulting from both reduced proliferation and accelerated cell death.

**NO Production In Hyperglycemia**

NO is the major vasodilator and key survival factor of the endothelium. Reduction in NO production caused by a reduced eNOS expression or phosphorylation at the site Ser\textsuperscript{1177} is associated with impaired proliferation and an increase in apoptosis, possibly contributing to the development of endothelial dysfunction and atherosclerosis in diabetes mellitus.\textsuperscript{31–33} In the present study, we demonstrate that hyperglycemia results in the induction of PP2A, which contributes to a decline in eNOS phosphorylation and NO liberation from CPCs. The impaired eNOS-derived NO release may play a role in attenuated VEGF-mediated vasculogenesis, because NOS inhibition by L-NAME was recently shown to lower serum VEGF levels.\textsuperscript{9} Moreover, hypoxia-induced upregulation of VEGF activates eNOS predominantly through AKT-dependent eNOS phosphorylation, which in turn promotes an increase in circulating CPCs.\textsuperscript{10,11} In case of hyperglycemia, the PP2A-mediated reduction of eNOS phosphorylation at Ser\textsuperscript{1177} might lead to a blunted response to an hypoxia-induced upregulation of VEGF and, hence, an impairment of vasculogenesis in hyperglycemic patients.

Another mechanism regulating eNOS phosphorylation involves the protein kinase AKT, which is impaired by HG, as shown by our data. Based on previous reports, demonstrating the involvement of PP2A in the regulation of AKT,\textsuperscript{8} we hypothesized that PP2A might also be involved in HG mediated impairment of AKT activity. However, as demonstrated by our results, PP2A inhibition does not completely restore AKT activity in HG. We therefore conclude that PP2A-independent, HG-mediated mechanisms exist, which yet have to be determined.

**Influence of Hyperglycemia on Migratory and Integrative Capacity**

Several studies investigating the function of CPC/endothelial progenitor cells demonstrate an impaired infiltrative and integrative capacity of CPC from diabetic patients.\textsuperscript{15,16} Based on the reported data, this reduced infiltrative/integrative capacity of CPC after culture in hyperglycemic medium seems to be the result of a diminished MMP-9 activity. MMP-9 has been shown to stimulate proliferation and release of endothelial and hematopoietic stem cells from the bone marrow\textsuperscript{34–35} and to promote angiogenesis.\textsuperscript{36}

The regulation of migratory and infiltrative capacities of cells, as well as MMP-9 activity by local NO levels, are discussed contradictory in the literature. Both decreased and increased NO levels have been described to impair MMP-9 activity, as well as cell migration.\textsuperscript{9,37–40} These contradictions might result from the use of different cell types, NO concentrations/NOS inhibitors, and read-out parameters used in the respective reports. Because NO is regarded as a survival factor, as well as an inducer of apoptosis, depending on its local concentration, the same might be true for its role in mediating tissue infiltration by different cell types like inflammatory cells or endothelial cells. We report in the present study a decline in MMP-9 activity and invasive/integrative activity of the CPCs only in the control cells, but not in the HG group. Therefore, the decrease in MMP-9 activity and ECM gel invasion might be mediated by the decrease in NO production seen in HG.

In conclusion, our findings indicate that hyperglycemia leads to a loss of functional competence in CPCs. Projected to the situation in the vasculature of diabetic patients, this might imply that even if CPCs find a target in the damaged endothelium, the dysfunctional endothelial cell will be replaced by a dysfunctional CPC. The CPCs might not be able to produce proper amounts of NO to mediate a physiological vasodilation to respond adequately to VEGF stimulation and to promote vasculogenesis.

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**References**


Hyperglycemia Reduces Survival and Impairs Function of Circulating Blood-Derived Progenitor Cells

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