Hyperglycemia Accelerated Endothelial Progenitor Cell Senescence via the Activation of p38 Mitogen-Activated Protein Kinase

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Background Both the number and function of bone marrow-derived endothelial progenitor cells (EPCs) have been shown to be impaired in patients with diabetes mellitus. Therefore, we investigated the effect of glucose on the senescence of EPCs.

Methods and Results EPCs were isolated from human peripheral blood and characterized to evaluate the effect of glucose (in 5–12.5 mmol/L) on the rate of senescence by acidic β -galactosidase staining. The phosphorylation of p38 mitogen-activated protein kinase (MAPK) level was analyzed by ELISA. The exposure of cultured EPC to high glucose (HG; 12.5 mmol/L) significantly accelerated the rate of senescence compared with that in osmolar control (L-glucose) during 10 days culture. An inhibitory effect of HG on EPC proliferation disclosed by an MTS assay. The phosphorylation of p38 MAPK in EPCs was also increased by glucose compared with control in a dose-dependent manner. HG-induced EPC senescence was significantly inhibited by the addition of an inhibitor of the p38 MAPK, SB203580.

Conclusions HG accelerates the onset of EPCs senescence leading to the impairment of proliferative activity, which might be related to the phosphorylation of p38 MAPK. (*Circ J* 2006; 70: 1076–1081)

Key Words: Endothelial progenitor cell; Glucose; p38 mitogen-activated protein kinase; Senescence

Cardiovascular complications based on atherosclerosis are the leading causes of morbidity and mortality in patients with diabetes mellitus (DM), and the endothelial dysfunction might be the beginning of the atherosclerosis.^{1–3} 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.² All forms of diabetes are characterized by chronic hyperglycemia and the development of diabetes-specific microvascular pathology in the retina, renal glomerulus and peripheral nerve.

Recent studies indicate that elevated glucose concentration can induce dysfunction of several intracellular signal transduction cascades, including modulation of protein kinase C, generation of reactive oxygen species and accumulation of advanced glycation end products.⁴ Furthermore, it has been reported that high glucose (HG) levels can accelerate the p38 mitogen activated protein kinase pathway in endothelial cells.⁵ Mitogen-activated protein kinases (MAPKs) regulate cellular proliferation and differentiation induced by a variety of cellular stresses. MAPKs are the family of serine/threonine kinases that are divided 3 to major subgroups: extracellular signal-regulated kinase (ERK); c-jun N-terminal kinase; and p38 MAPK.

Recent insights suggest that the injured endothelial monolayer is regenerated by circulating bone marrow

(BM)-derived endothelial progenitor cells (EPCs), which accelerate reendothelialization and limits atherosclerotic lesion formation.^{6–10} This beneficial property of BM-EPCs makes them attractive for cell therapy targeting the regeneration of ischemic tissue. In contrast, a reduction in circulating EPCs has been demonstrated in the presence of various risk factors for coronary artery disease (CAD), such as hyperlipidemia, hypertension, smoking, chronic renal failure, aging and DM.^{11–17} Whether such risks affect the BM production of EPCs or impair the function of EPCs remains to be determined.

Based on this information, we hypothesized that hyperglycemia may impair the function of BM-EPCs through p38 MAPK pathway. We further examined the effect of glucose on the senescence of EPCs. In addition, we investigated whether these effects would be improved by SB203580, an inhibitor of p38 MAPK.

Methods

Isolation of Mononuclear Cells (MNCs) and Cell Culture

EPCs were cultured according to a previously described technique.^{18,19} Briefly, peripheral blood (PB)-MNCs were isolated from healthy volunteers using density gradient centrifugation with a Histopaque 1077 (Sigma Chemical Co, St Louis, MO, USA). After purification with 3 washing steps, 10×10^6 PB-MNCs were plated on fibronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2) (Clonetics; Walkersville, MD, USA) with EGM-2MV single aliquots containing 5% fetal bovine serum, vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor and ascorbic acid. EPCs were treated with glucose in differ-

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Fig 1. Glucose inhibits endothelial progenitor cells (EPCs) differentiation in human mononuclear cells (MNCs). MNCs were incubated with different glucose concentrations (5, 7.5, 10, 12.5 mmol/L) for 4 days. Representative pictures of dual-stained EPCs (lectin (green) and acetylated low-density lipoprotein (red)) from control cultures (A) and high glucose (HG) cultures (B) after 4 days. The number of differentiated, adherent EPCs in HG condition (D-glucose 12.5 mmol/L) revealed significant decrease compared with control in a dose-dependent manner (C). Data are mean $\pm$ SEM, $n=5$, * $p<0.01$ vs control. LPF, low-power fields.

ent concentrations (5, 7.5, 10 and 12.5 mmol/L). L-glucose was used as an osmolar control because it is known to have no impact on glucose metabolism. SB203580 (1, 10 μ mol/L, Promega, Madison, Wisconsin, USA), an inhibitor of p38 MAPK, was applied to hyperglycemic media containing 12.5 mmol/L D-glucose. After 4 days of culture, adherent cells were washed with medium and incubated with 2.4 g/ml 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (acLDL) for 1 h. Cells were fixed in 2% paraformaldehyde for 10 min and lectin staining was performed by incubation with isothiocyanate-labeled Ulex Europaeus agglutinin I (lectin; 10 μ g/ml; Sigma) for 1 h. After staining, samples were viewed under an inverted fluorescent microscopy (Olympus, Tokyo, Japan). Dual-stained cells that were positive for lectin and acLDL were judged to be EPCs and these cells were counted per well. The number of EPCs per well was evaluated by counting 3 randomly selected low-power fields.

Senescence-Associated -Galactosidase (SA- -gal) Activity Assay

EPCs were harvested 4 days after culture and re-seeded. Following adding of several concentrations of glucose into EPCs, EPCs at day 7 were harvested and then SA- -gal activity was measured as previously described¹⁸. Briefly, EPCs were washed in phosphate buffered saline, fixed for 3 min (room temperature) in 2% paraformaldehyde, washed and incubated for 24 h at 37°C (no CO₂) with fresh a SA- -gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indyl -D-galactopyranoside, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, 2 mmol/L MgCl₂, 0.01% sodium deoxycholate and 0.02% Nonidet-40). EPCs were counterstained with 4',6-diaminophenylindole (0.2 μ g/ml in 10 mmol/L Tris-HCL, pH 7.0, 10 mmol/L EDTA, 100 mmol/L NaCl) for 10 min to count the total cell number.

Proliferative Activity Assay

Mitogenic activity was assayed using a colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96

AQ; Promega, Madison, WI, USA). EPCs were harvested 4 days after culture and re-seeded with a 96-well plate (1 $\times$ 10⁴ cells) in 0.1 ml of EBM-2 medium. After 7 days in culture, MTS/phenazine methosulfate solution was added to each well for 3 h, whereupon light absorbance at 490 nm was detected using an ELISA plate reader (Bionetics Laboratory, Kensington, MD, USA).

Measurement of MAPKs

For the analysis of the phosphorylation of either p38 MAPK or ERK1/2, a Cellular Activation of Signaling ELISA (CASE™) kit (SuperArray Bioscience Corp, Frederic, MD, USA) was used according to the manufacturer's protocol. In the CASE assay, EPCs were re-seeded onto a 96-well plate and cultured for 7 days. The cells were fixed with 4% formaldehyde to preserve any phosphorylation modification. Both anti-phospho-protein specific antibody (Ab) and anti-pan-protein specific Ab were used as primary Abs. Following incubation with primary and secondary Abs, the amount of bound protein in each well was determined using a developing solution and an ELISA Plate Reader. The absorbance readings were then normalized to the relative cell number as determined by cell staining and an ELISA Plate Reader. The amount of phosphorylated protein, after normalization to the amount of total protein, was then used directly as an index of the degree of activation of the downstream pathway.

Statistical Analysis

Data were expressed as mean $\pm$ SEM based on at least 5 independent experiments. Statistical analysis was performed by 1-way analysis of variance (analysis of variance test) for multiple testing. Probability values were considered significant at $p<0.05$.

Results

Glucose Inhibits EPCs Differentiation in Human MNCs

The EPCs were characterized as adherent cells that were

Fig 2. High glucose accelerates endothelial progenitor cells (EPCs) senescence. EPC senescence was evaluated by senescence-associated β -galactosidase (SA- β -gal) staining. Representative photomicrographs show SA- β -gal-positive cells (blue) in EPCs treated with different glucose concentrations (A: 5 mmol/L; B: 12.5 mmol/L). (C) At day 7 after culture, the number of SA- β -gal positive cells was counted manually from a total of 200 cells. Data are expressed as mean $\pm$ SEM (n=5). *p<0.01 compared with control.

Fig 3. Glucose impairs mitogenic activity in endothelial progenitor cells (EPCs). At day 7 after culture, the mitogenic activity was detected as described in the methods. Data are expressed as mean $\pm$ SEM (n=5). *p<0.01 compared with control.

double positive for both lectin and acLDL uptake. We examined the effect of glucose on EPCs differentiation. MNCs were incubated with different glucose concentrations (5, 7.5, 10 and 12.5 mmol/L) for 4 days. The number of differentiated, adherent EPCs in HG condition revealed significant a decrease compared with controls in a dose-dependent manner. L-glucose, as an osmolar control, did not affect EPC differentiation (Fig 1).

Glucose Accelerates EPCs Senescence

To assess the onset of senescence, acidic β -galactosidase was detected as a biochemical marker for acidification for the onset of cellular senescence. Co-incubation with glucose accelerated the increase in SA- β -Gal-positive cells dose-dependently (Figs 2A,B).

Glucose Impairs Mitogenic Activity in EPCs

Having demonstrated that glucose accelerated the onset of senescence, we examined whether it translated into an impairment of mitogenic activity in EPCs. MTS assay disclosed a significantly inhibitory effect of glucose on EPC proliferation at day 7 (Fig 3).

Glucose Induces Phosphorylation of MAPKs

The phosphorylated levels of MAPKs in EPCs were evaluated using CASE assay. Incubation of EPCs with glucose increased p38 and ERK1/2 activity dose-dependently (Fig 4).

SB203580 Increases the Number of EPCs and Reduces Glucose-Induced EPC Senescence

Having demonstrated that glucose increases p38 activity, we investigated the effect of the p38 inhibitor on EPCs differentiation in human MNCs. SB203580 significantly recovered glucose-induced reduction of a number of EPCs (Fig 5). Likewise, the glucose-induced increase in SA- β -Gal-positive cells was significantly attenuated through treatment with SB203580 (Fig 6). In contrast, PD 98059, an inhibitor of ERK 1/2, had no effect on HG-induced EPC senescence.

Discussion

Recent studies have provided increasing evidence that the functional regeneration of ischemic tissue by improved neovascularization and possibly tissue repair is critically

Fig 4. Effect of glucose on mitogen-activated protein kinases (MAPKs) phosphorylation in endothelial progenitor cells (EPCs). (A) Incubation of EPCs with glucose dose-dependently increased p38-phosphorylation. The levels of phosphorylated p38 MAPK in the control cells were defined as 100%. (B) Glucose activated the MAPK extracellular signal-regulated kinase (ERK)1/2 dose-dependently. Data are expressed as mean $\pm$ SEM (n=5). *p<0.01 compared with control.

Fig 5. SB203580 increases the number of endothelial progenitor cells (EPCs). Treatment with SB203580 prevented high glucose (HG)-induced reduction of the number EPCs. Data are expressed as mean $\pm$ SEM (n=5). *p<0.01 compared with control; #p<0.01 compared with HG.

Fig 6. SB203580 reduces glucose-induced endothelial progenitor cell senescence. The high glucose (HG)-induced increase in senescence-associated β -galactosidase-positive cells was significantly attenuated by the co-treatment with SB203580. Data are expressed as mean $\pm$ SEM (n=5). *p<0.01 compared with control; #p<0.01 compared with HG.

dependent on the mobilization and integration of EPC into the ischemic tissue. There are no specific studies in the literature on therapeutic revascularization with cell therapy for diabetic patients, although many studies have demonstrated that diabetic patients displayed poorer responses to cell therapy than non-diabetic patients.^{20–30} Indeed, Tamarat et al demonstrated a reduced angiogenic potential of EPCs in diabetic animals. Administration of diabetic progenitors into the ischemic hindlimbs of non-diabetic mice improved angiogenic response to a lesser extent.³¹ Moreover, Tepper et al reported that the proliferation and tube formation of EPCs were impaired in patients with type 2 diabetes compared with normal subjects.³² Although these studies have clarified an adverse effect of DM on the functional activity of EPCs, the underlying mechanisms remain unsolved.

While, aging or senescence may constitute a potential limitation to the ability of progenitor cells to sustain ischemic tissue and repair. Edelberg et al demonstrated that only the transplantation of young BM-derived cells restored age-associated impaired neovascularization, whereas the BM of aged mice was not effective.³³ Moreover, the migratory capacity of PB-derived EPCs of elderly patients with CAD is significantly reduced.^{11,34} These findings highlight a potentially relevant feature of the endogenous repair process and poses an interesting question on the value of therapies based on the increase of endogenous EPC in either elderly patients or patients with atherosclerotic risk factors.

The present study demonstrates that HG induces a reduction of the number of EPCs. Moreover, inhibition of p38 MAPK restored the antiangiogenic effects of hyperglycemia. The mechanisms by which glucose accelerates the onset of EPC senescence remains to be determined. It has been reported that glucose can activate the p38 MAPK pathway in many cell types, including EPCs.^{6,35} Several studies demonstrate that p38 MAPK blockage is associated with increased angiogenesis.^{36–38} Keeping these findings in mind, we speculated that glucose accelerates EPC senescence through p38 activation. However, further studies will be needed to clarify our speculation.

In conclusion, we provide evidence that glucose inhibits EPC differentiation via the p38 MAPK pathway. The inhibition of EPC senescence may importantly improve the functional activity of EPCs for potential cell therapy. p38 MAPK has a critical role in EPCs senescence and may represent a sensitive target in a number of angiogenesis-related disorders including DM.

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