High glucose impairs the proliferation and increases the apoptosis of endothelial progenitor cells by suppression of Akt

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ABSTRACT

Aims/Introduction: Endothelial progenitor cells (EPC) play a critical role in adult vasculogenesis and vascular repair. Previous studies have described the dysfunction of EPC in diabetic patients, but the precise mechanism is still unclear. To elucidate the dysfunction of EPC in diabetic patients, we investigated the functions and intracellular signaling of EPC under normal or high glucose conditions. We also examined the number of EPC in the peripheral blood of Japanese type 2 diabetic patients.

Materials and Methods: EPC were cultured with normal or high glucose. Subsequently, the proliferation and the apoptosis of EPC were assessed in the presence or absence of vascular endothelial growth factor (VEGF). The phosphorylation of Akt was assessed by western blot analyses. We compared the number of CD34⁺CD45⁻ progenitor cells, which is considered as a marker of EPC in non-diabetic and type 2 diabetic subjects, using flow cytometry.

Results: High glucose decreased the proliferation of EPC and increased the number of apoptotic cells. VEGF significantly increased the proliferation and suppressed the apoptosis of EPC, both of which were abolished by PI 3-kinase inhibitor, LY294002. High glucose significantly suppressed the basal and VEGF-stimulated phosphorylation of Akt in EPC. Furthermore, the number of circulating EPC was decreased in type 2 diabetic patients, although there were no significant differences in the serum levels of VEGF between control subjects and diabetic patients.

Conclusions: These findings suggest that high glucose impairs the functions of EPC through the suppression of Akt phosphorylation stimulated by VEGF.

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KEY WORDS: Akt, Endothelial progenitor cells, Vascular endothelial growth factor

INTRODUCTION

Endothelial progenitor cells (EPC) play an important role in postnatal neovascularization and vascular repair. Circulating EPC adhere to the sites of vascularization and migrate into the target tissue resulting in the growth of new blood vessels. Previous experimental studies have shown successful results of the transplantation of EPC in targeted ischemic tissues. A number of preclinical studies on the transplantation of EPC have been carried out in the past decade. The safety of EPC transplantation is thought to be established, although the effects are modest. EPC exist in bone marrow, cord blood and in small numbers in peripheral blood and local tissue. Previous studies have provided evidence that decreased numbers and activities of EPC might contribute to impaired vascularization in patients with coronary artery diseases. The EPC levels were negatively correlated with the degree of carotid stenosis, graft vasculopathy and tissue ischemia. Patients with risk factors for coronary artery diseases, such as diabetes, dyslipidemia and hypertension, are also reported to have decreased and dysfunctional EPC. These findings suggest that the dysfunction of EPC might affect not only the efficacy of transplantation therapy with autologous EPC, but also vascular functions.

An essential diabetic complication is the vascular dysfunction of micro- and macrovasculatures. From this viewpoint, the dysfunction of EPC might advance the progress of diabetic complications. In fact, impaired functions of EPC were reported in patients with proliferative diabetic retinopathy. To elucidate the mechanisms of the diabetes-induced dysfunction of EPC is important for better management of diabetic complications.

Vascular endothelial growth factor (VEGF) increases the proliferation and migration of endothelial cells. The anti-apoptotic effects of VEGF are also reported to be important in
angiogenesis. VEGF affects not only mature endothelial cells, but also EPC. VEGF induces the mobilization of bone marrow-derived EPC, resulting in the increase of differentiated EPC and neovascularization. To elucidate the mechanisms by which diabetes interferes with EPC, we investigated the growth and apoptosis of EPC under normal or high glucose conditions and examined the proliferative and anti-apoptotic effect of VEGF. In addition, we examined the number of EPC in the peripheral blood of Japanese type 2 diabetic patients.

MATERIALS AND METHODS

Human Umbilical Cord Blood

Human umbilical cord blood was obtained from each donor after the baby's delivery. Written informed consent was obtained from all mothers before labor and delivery. Protocols for sampling human umbilical cord blood were approved by the Institutional Review Board.

Isolation and Culture of EPC

Isolation and ex vivo expansion of EPC were carried out as previously described. In brief, cord blood-derived mononuclear cells from human volunteers were plated on human fibronectin-coated (Sigma, St. Louis, MO, USA) culture dishes and maintained in Medium 199 (Sigma) supplemented with 20% ES-qualified fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). After 4 days in culture, non-adherent cells were removed by washing, new media was applied and the culture was maintained through to day 7. EPC were identified by the surface markers CD34, KDR, CD31 and Tie2, using flow cytometry.

Proliferation Assay

Seven days after isolation, $2 \times 10^4$ EPC were seeded in each six-well plate for the growth assay. EPC were cultured with Medium 199 containing 5.5 mmol/L glucose (normal glucose conditions) or 20 mmol/L glucose (high glucose conditions) and 2% FBS for 72 h. After the incubation, the numbers of EPC were counted.

DNA synthesis was measured by a Cell Proliferation 5-bromo-2-deoxyuridine (BrdU) ELISA assay (Roche Diagnostics, Mannheim, Germany). Briefly, $1 \times 10^4$ cells were plated with Medium 199 containing 5.5 mmol/L glucose (normal glucose conditions), 20 mmol/L glucose (high glucose conditions)
or 5.5 mmol/L glucose + 14.5 mmol/L L-glucose (L-glu) as an osmotic control and 2% FBS for 72 h in 96-well plates. In the case of VEGF (human recombinant VEGF; R&D Systems, Minneapolis, MN, USA) stimulation experiments, VEGF was added 24 h before the BrdU assay. PI 3-kinase inhibitor, LY294002 (2 μmol/L) was pretreated 30 min before VEGF stimulation. The BrdU ELISA was carried out according to the manufacturer’s standard procedure and the absorbance was read at 405 nm on a Wallac 1420 ARVO microplate reader (Aloka, Tokyo, Japan).

Detection of Apoptosis
The numbers of apoptotic EPC induced by the high glucose condition were detected using Hoechst 33342. The adherent EPC were maintained in Medium 199 containing 5.5 mmol/L glucose (normal glucose conditions) or 20 mmol/L glucose (high glucose conditions) and 2% FBS with or without 25 ng/mL of VEGF for 72 h. LY294002 (2 μmol/L) was pretreated before VEGF stimulation. After incubation, cultured EPC were washed with PBS and detached with trypsin-EDTA. After centrifugation, the cell pellet was suspended at 1–3 × 10⁴ cells/mL in 50 μL of PBS and stained with Hoechst 33342. Apoptotic cells were defined as those with condensed nuclear chromatin. The percentages of apoptotic cells to total live cell counts were calculated from 10 random views.

Western Blot Analysis
The phosphorylation of Akt stimulated by VEGF under normal or high glucose conditions was examined by western blot analysis. Confluently-grown EPC were starved with Medium 199 containing 0.5% FBS for 24 h, then stimulated with VEGF (25 ng/mL) for the indicated periods. Cells were lysed in 100 μL of lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L phenylmethysulfonyl fluoride, 10 μg/mL aprotinin, 50 μg/mL leupeptin, 1 μg/mL pepstatin A and 1 mmol/L Na₂VO₃). A sample (20 μg) of lysate protein was subjected to SDS-PAGE under reducing conditions and immunoblotting. The antibodies used for immunoblot analyses were as follows: anti-phospho-Akt (Ser473), anti-Akt (Cell Signaling Technology, Beverly, MA, USA). The primary antibodies were added at a dilution of 1:1000 overnight at 4°C. After washing, the appropriate secondary antibodies were added at a dilution of 1:1000 for 1 h at room temperature. Blots were developed with super signal enhanced chemiluminescence kits and were visualized by chemiluminescence with Image Master.

Quantification of Circulating Endothelial Progenitor Cells
Peripheral blood samples were collected from type 2 diabetics (n = 59) or non-diabetic control subjects (n = 72) in Nagoya University Hospital and Aichi Gakuin University Dental Hospital. The study protocol and informed consent procedures were approved by the Ethics Committees of Nagoya University Graduate School of Medicine, and School of Dentistry, Aichi Gakuin University. Circulating CD34⁺CD45⁻low progenitor cells, which are considered as EPC, were counted using flow cytometry analysis as previously described39. Mononuclear cells in peripheral blood were separated with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). The buffy coat was washed with...
PBS containing 10% FBS. Cells were labeled with allophycocyanin (activated protein C)-conjugated anti-CD45 monoclonal antibody (Invitrogen) and PC5-conjugated anti-CD34 monoclonal antibody (Beckman Coulter, Brea, CA, USA), whereas FITC-conjugated anti-mouse F(ab)2 (Invitrogen) was used as a secondary antibody and analyzed by flow cytometry (FACS Calibur; BD Biosciences, Franklin Lakes, NJ, USA). After appropriate gating with low cytoplasmic granularity and with low expression of CD45, the numbers of CD34 positive cells were quantified and expressed as number of cells per 10^6 total events.

**Measurement of Serum Concentration of VEGF**

A fasting venous blood sample was collected. After centrifugation at 4°C, the serum fraction was collected. VEGF165 levels were estimated using the ELISA system according to the manufacturer’s instructions (R&D Systems).

**Statistical Analyses**

Statistical analyses were carried out using SPSS for Windows version 12.0 (SPSS, Chicago, IL, USA). All the group values were expressed as means ± SE. Significance was defined as a $P$-value <0.05.

**RESULTS**

**Identification of EPC**

EPC, isolated from the culture of cord blood-derived mononuclear cells, expanded from the attached cells. More than 90% of the isolated cells were identified by DiI-acetylated LDL, which is a marker of vascular endothelial cells (data not shown).

**Figure 4** Detection of apoptosis in endothelial progenitor cells (EPC) cultured under the normal or high glucose condition. (a) The apoptotic cells (arrows indicate) under 5.5 mmol/L glucose (NG) and 20 mmol/L glucose (HG) conditions were visualized with the staining of the cells with Hoechst 33342. (b) The adherent EPC were maintained in serum starved-Medium 199 containing NG or HG with or without 25 ng/mL of vascular endothelial growth factor (VEGF) for 72 h. LY294002 was pretreated before VEGF stimulation. Apoptotic cells were detected by staining with Hoechst 33342. Numbers of apoptotic EPC are the mean ± SE percentage of total cells. *$P$ < 0.01, **$P$ < 0.05 vs normal glucose control.
Flow cytometric analyses showed positive staining with CD34 (88.6%), CD31 (90.5%), KDR (72.7%) and Tie-2 (77.2%; Figure 1).

**Proliferation and Apoptosis of EPC Under the Normal and High Glucose Conditions**

The proliferation of EPC was evaluated by the number of cells and DNA synthesis. EPC were cultured under the normal or high glucose condition for 72 h. The number of EPC was 53% lower under the high glucose condition compared with that under the normal glucose condition (normal glucose, 6.8 x 10^4 cells/well; high glucose, 3.2 x 10^4 cells/well, P < 0.05; Figure 2).

DNA synthesis of EPC was 38% lower under the high glucose condition compared with that under the normal glucose condition (P < 0.01 vs 5.5 mmol/L glucose; Figure 3). Under the l-glucose hyperosmotic condition, DNA synthesis was not changed compared with that under the normal glucose condition. VEGF significantly increased DNA synthesis under the normal and l-glucose hyperosmotic condition (P < 0.05 vs VEGF (-) in each group), which was abolished by PI 3-kinase inhibitor, LY294002. High glucose suppressed the effect of VEGF on DNA synthesis.

The apoptotic cells were detected by staining with Hoechist 33342 (Figure 4a). With high glucose, 33.4% of total cells were apoptotic EPC, whereas 7.9% of total cells were apoptotic under the normal glucose condition (Figure 4b). VEGF suppressed the apoptosis of EPC both under the normal and high glucose conditions (normal glucose condition, 32.7% [P < 0.05]; high glucose condition, 58.7% [P < 0.01]). Although VEGF suppressed the high glucose-induced apoptosis, the percentage of apoptotic cells was still 1.9-fold higher under the high glucose condition compared with the normal glucose condition (with VEGF under the high glucose condition, 13.7%; with VEGF under normal glucose condition, 7.9%; P < 0.05). In contrast, the inhibitory effect of VEGF on apoptosis was completely eliminated by LY294002 both under the normal and the high glucose conditions.

**Phosphorylation of Akt in EPC Under the Normal or High Glucose Condition**

Because Akt is a key molecule in cell growth and survival, we evaluated the phosphorylation of Akt after 72 h of culture under the normal or high glucose condition. Western blot analysis showed that high glucose significantly decreased the phosphorylation of Akt by 27% compared with that under the normal glucose condition (P < 0.05; Figure 5).

**VEGF-Stimulated Akt Signaling Under the High Glucose Condition**

To evaluate the intracellular signaling of VEGF, we examined the effect of VEGF on Akt phosphorylation under the normal or high glucose condition. VEGF stimulated the phosphorylation of Akt in a time-dependent manner, both under the normal and high glucose condition (Figure 6). However, the maximum effect of Akt phosphorylation was significantly lower under the high glucose condition compared with that under the normal glucose condition (normal glucose, 3.1 ± 0.1-fold; high glucose, 2.0 ± 0.1-fold, P < 0.05). We also examined the effect of VEGF on Akt phosphorylation under 40 mmol/L high glucose conditions, although this is an over-physiological condition in vivo. Akt phosphorylation was completely suppressed under the 40 mmol/L high glucose condition (Figure S1).

**Circulating CD34⁺CD45⁻ Progenitor Cells in Age-Matched Non-Diabetic Subjects and Type 2 Diabetic Patients**

Circulating EPC were counted as CD34⁺CD45⁻ progenitor cells by surface markers using flow cytometry analysis in age-matched non-diabetic subjects and type 2 diabetic patients (Figure 7). The clinical characteristics are shown in Table 1. Bodyweight, body mass index, fasting blood glucose and HbA1c were significantly higher in type 2 diabetic patients than in non-diabetic control subjects. As shown in Figure 8, the number of CD34⁺CD45⁻ progenitor cells in peripheral blood was significantly decreased in type 2 diabetic patients by 14.2% (P < 0.05). No significant differences in the serum concentrations of VEGF were observed between control subjects and type 2 diabetic patients.

Next, we examined the correlation coefficients of clinical data with the number of CD34⁺CD45⁻ progenitor cells (Table 2). Data were normally distributed and suitable for parametric
analysis. Pearson's correlation coefficients were calculated using the SPSS statistics package. In the present study, HbA1c and the number of CD34+CD45low progenitor cells were significantly and negatively correlated ($r = -0.265, P = 0.004$).

**DISCUSSION**

Clinical trials of transplantation using mononuclear cells from bone marrow or peripheral blood that included EPC have been carried out for the treatment of ischemic diseases.$^{20,21}$ The
investigation on EPC functions showed that EPC play an important role not only in vasculogenesis, but also in vascular repair. In the present study, we showed that high glucose suppressed Akt phosphorylation of EPC, which might result in increased apoptosis and decreased growth in EPC. VEGF-stimulated Akt phosphorylation was also inhibited under the high glucose condition. The number of EPC was decreased in type 2 diabetic patients, although there were no significant differences in the serum levels of VEGF between control subjects and diabetic patients. These results suggest that high glucose impairs EPC functions through the inhibition of Akt.

There is increasing evidence that a decrease in the number and functional activities of EPC is closely associated with cardiovascular deficits. The decreased number of EPC might predict death from cardiovascular causes. The number and functions of EPC are also decreased in patients with cardiovascular risk factors including diabetes. We have shown a negative correlation between HbA1c and the number of EPC in Japanese type 2 diabetic patients, which is consistent with previous studies in type 1 and type 2 diabetic patients of different races. These results suggest that high glucose directly affects the number of EPC.

VEGF prevents the apoptotic death of endothelial cells during angiogenesis or experimental hypoxia. VEGF also mediates the survival of immature vessels through KDR via the PI3-K/Akt pathway. Akt, which is located downstream of PI3-kinase, is an essential regulator of various cellular processes, including glucose metabolism and cell survival. Several studies have shown that VEGF promotes cell growth and inhibits apoptosis through Akt. We have shown the anti-apoptotic and proliferative effects of VEGF in EPC through Akt. Our observations are consistent with a previous study showing that VEGF gene transfer to EPC increases vasculogenesis in EPC transplantation to ischemic tissues. High glucose impaired the anti-apoptotic and proliferative effects of VEGF in EPC. We have shown that human EPC isolated from cord blood have impaired VEGF-stimulated phosphorylation of Akt and increased apoptosis by high glucose, which are consistent with the results of EPC in diabetic miniswine by another group. We have not examined other VEGF signaling pathways, such as ERK, nor p38 MAP kinase. Further studies are required to clarify the impacts of each signaling pathways of VEGF in EPC. However, the present results suggest that the suppression of Akt signaling under the high glucose condition might be one of the causes of EPC dysfunction in diabetic patients.

In summary, the present study showed that high glucose impairs Akt signaling in EPC, followed by their decreased proliferation and increased apoptosis. The anti-apoptotic functions of VEGF in EPC are important for preventing atherosclerosis. However, VEGF-Akt signaling is also impaired under the high glucose condition. These results suggest that hyperglycemia in diabetic patients might not only reduce the efficacy of transplantation therapy with autologous EPC for ischemic tissues, but also cause vascular dysfunction resulting in the progression of diabetic complications.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** | The phosphorylation of Akt stimulated by vascular endothelial growth factor (VEGF) under 40 mmol/L high glucose condition (HG).

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