

Exogenous FGF-2 improves biological activity of endothelial progenitor cells exposed to high glucose conditions

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ABSTRACT

Purpose: To investigate the effects of exogenous basic fibroblast growth factor -2 (FGF-2) on the biological activity of endothelial progenitor cells (EPCs) exposed to high glucose conditions.

Materials and Methods: 1) Bone marrow EPCs from C57BL/6 mice were isolated and cultured in vitro. EPC purity was identified by flow cytometry and immunofluorescence staining. 2) Apoptosis was detected by TUNEL assay. Migration and tube formation ability was detected by Transwell chamber and Matrigel assays, respectively. The expression and activation of β -catenin was detected by Western blot. 3) Doppler flowmetry was used to detect the effect of FGF2 on blood flow recovery in ischemic hind limbs of mice.

Results: 1) FGF-2 treatment reversed high glucose induced growth inhibition of EPCs. FGF-2 treatment also increased migration and tube formation ability of EPCs even in high glucose conditions. 2) Western blot analysis demonstrated that the percentage of activated β -catenin/total β -catenin in the high glucose group were significantly lower than that in the control group, while FGF-2 treatment reversed high glucose induced β -catenin inhibition. 3) In vivo experiments demonstrated that the blood flow recovery in ischemic hind limbs of mice was significantly improved after FGF-2 treatment.

Conclusion: Exogenous FGF-2 could play a role in the functional repair of damaged EPC exposed to high glucose conditions, via the activation of the Wnt/ β -catenin signaling pathway.

Keywords: fibroblast growth factor-2; endothelial progenitor cells; angiogenesis; wnt/ β -catenin signaling pathway; high glucose environment

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INTRODUCTION

Endothelial progenitor cells (EPCs) play an important role in the microcirculation reconstruction of ischemic regions, and have a curative effect in reversing ischemia in lower limb ischemic mouse models (1). However, high glucose induced oxidative stress impairs the function of autologous EPCs in diabetic patients with hyperglycemia. Therapies are needed to improve and promote angiogenesis of EPCs and increase microcirculation, especially in diabetic patients with lower limb ischemia. Basic fibroblast growth factor -2 (FGF-2) is a pleiotropic peptide which can regulate cell division, proliferation, migration and differentiation. It can also promote angiogenesis, wound healing, embryonic development and organ differentiation (2). However, the effect of high glucose or hyperglycemia and its

effect on FGF-2 and EPC dysfunction have not been studied. Thus, our study focuses on the effects of exogenous FGF-2 on EPC function and recovery after exposure to high glucose conditions.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6J mice (purchased from Shanghai Slack Experimental animal breeding Co. Ltd.) were used in this study. All mice were housed in SPF, with 12 h day and night cycle. All animal experiments were approved by the Shanghai Animal Ethics Committee.

Reagents

All reagents were purchased from the following companies; EGM2 Culture medium (Cloneties, USA); fetal bovine serum (US Gibco Corporation); Trypsin-EDTA; TUNEL work fluid (Roche Corporation); Matrigel (US Becton Dickson Corporation); Monoclonal antibodies CD31, CD144, CD45, and KDR (US Abcam Corporation); FITC Ulex lectin I (FITC-UEA-I, Sigma); Dil labeled acetylated low-density lipoprotein (Dil-ac-LDL, Molecular Probe); 0.25% trypsin (US Invitrogen Corporation); paraformaldehyde and RIPA lysate (Shanghai bio engineering); BCA protein concentration assay kit (azure sky biology); IgG Goat anti rabbit two anti (Wuhan, Doctor De, biology); ECL fluorescent display agent (US Pierce Corporation); Rabbit anti mouse

active β -catenin, β -catenin polyclonal antibodies (US Abcam Corporation).

All instruments used in the study are as follows; 24 orifice plates and Transwell chambers (US Corning Corporation); Flow cytometry (US BD Corporation); Fluorescence inverted microscope (Olympus, Japan); Gel image analysis software (Shanghai sky power company); Electrophoresis Tanks, electro rotating instruments and enzyme markers (Bio-Rad, USA); CO₂ cell incubator (US Thermo Corporation); Laser Doppler flowmetry (US Devon).

EPCs isolation, culture and identification

All mice were euthanized using cervical dislocation after pentobarbital administration. Bone marrow tissue was harvested from the femur and tibia under aseptic conditions and mononuclear cells were isolated. Cells were cultured in EGM2 medium, supplemented with VEGF growth factors and 5% fetal bovine serum. After three days of culture, media was changed and adherent cells were cultured at 37 °C and 5% CO₂. Media was changed every three days. EPCs cell phenotype was analyzed by flow cytometry. Dil-ac-LDL and FITC-UEA-I double fluorescence staining were used to identify EPCs.

EPC grouping and processing

After 7 days of culture, EPCs were harvested with 0.25% trypsin and cell suspensions with a density of 1×10^6 /mL were seeded into 6 well plates. The treatment groups were as follows; normal control group, high glucose group (30 mmol/L glucose) and high glucose + FGF-2 (30 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL and 300 ng/mL).. After 48hrs of treatment, phenotypic assays were performed. The cells in the normal control group were cultured in EGM2 medium (containing 5.5 mmol/L glucose, supplemented with VEGF, IGF and rhEGF added growth factor) + 5% fetal bovine serum. Cells in the high glucose group were cultured with similar components as the normal control group and 30 mmol/L glucose. . High glucose + FGF-2 treatment group were cultured similar to the high glucose group and with different concentration of FGF-2 (0, 30, 50, 100, 150, 300 ng/mL).

EPC apoptosis detection

TUNEL assay was used to determine apoptosis. Briefly, cells were fixed by 4% paraformaldehyde and stained with DAB and hematoxylin and then mounted in neutral balsam. The percentage of apoptosis cells was counted using 5 random fields under a high power microscope.

EPC cell migration assay

The migration ability of EPCs was evaluated by Transwell chambers. After synchronization, EPCs were digested using 2.5 g/L pancreatin and then resuspended in serum-free M199 medium. 2×10^4 cells were seeded in the upper chamber of 24 well-plates, while the bottom chamber contained media with 50 g/L VEGF. FGF2 was added on the upper chamber. The cells were incubated for 24 h, and then fixed with paraformaldehyde and stained with crystal violet. The number of EPCs that had migrated to the bottom chamber was counted. 6 randomly selected fields were used for assessment

using a high power microscope.

EPC cell formation assay

200ul of diluted Matrigel was added to pre cooled 24 well Matrigel slab and placed at 37 °C for 40 min for coagulation. Cells were collected using EGM-2 suspension, and each well was filled with 2×10^4 cells. The cells were incubated for 24hrs and tubular structures were counted under an inverted microscope.

Expression of activated β -catenin and total β -catenin by Western Blot Analysis

After 7 days of culture, cells were digested with 0.25% pancreatin, and cell suspensions were seeded at 1×10^6 mL⁻¹ with the different treatment conditions. After 48 h culture, total protein was extracted from the cells and protein concentrations determined by BCA assay. 20 μ g of protein were run on an SDS-PAGE gel, transferred to PVDF membrane and incubated with the relevant antibody. Bands were visualized using ECL chemiluminescent assay. Image-Pro Plus 6 software was used to determine and normalize OD band densities.

Hind limb ischemia mouse model establishment and Validation

Hind limb ischemia models were established using 8-week-old healthy male C57BL/6J mice. The following protocol was used to establish the model; First, mice were intraperitoneal anaesthetized with an injection of pentobarbital sodium (50 mg/kg), and both hind limbs were used to make the ischemic model. Second, mice were placed in a supine position, and the areas for surgery were disinfected. Longitudinal incisions from the groin to knee joint along the blood vessel lines were made using ophthalmic scissors. The femoral artery was exposed and the upper femoral artery was ligated. The femoral artery was dissected and removed from the femoral vein and nerve at the proximal location near the groin. Both the proximal and distal femoral artery was occluded and the skin was then sutured. Buprenorphine (0.04 mg/kg, SC) was used for postoperative analgesia. Peripheral blood for EPCs was harvested from each mouse. The mice with hind limb ischemia were randomly divided into four groups, with 6 mice in each group. The groups were designated into the; normal group, high glucose group, high glucose group+FGF2 (150 ng/mL). After 48 hours, the model was established. The different groups were tail vein injected with 1×10^7 EPCs, while one group was injected with cell-free culture medium as the control. Four weeks after the injection of EPCs cells, the mice hind limb blood perfusion condition was observed and measured using laser Doppler flowmetry. The red areas denoted rich areas of blood perfusion.

Statistical analysis

SPSS17.0 software was used to analyze the experimental data. Parametric data was analyzed using one-way analysis of variance (ANOVA) with Tukeys post hoc test for comparing multiple independent groups. Data was expressed as the mean \pm SEM. P < 0.05 denoted statistically significance.

RESULTS

EPC identification and cell culture conditions

The results showed that the cell morphology of EPC cells was oval in shape and demonstrated stable growth under culture conditions (Fig. 1AB). Adherent cells were observed using confocal laser scanning microscope. Cells were positive for Dil-Ac-LDL (red cytoplasmic fluorescence, (Fig. 1 C) and FITC-UEA-1

(green fluorescence (Figure 1 D)). Phenotypic analysis of EPCs using flow cytometry demonstrated the expression of relevant surface markers, ie antigen labeling were KDR, CD31, CD144, the percent of positive cells were $95.2 \pm 4.1\%$, $84.1 \pm 6.8\%$ and $77.5 \pm 7.8\%$, respectively. For the expression of hematopoietic stem cells using CD45, the percent of positive cells were $4.9 \pm 1.2\%$ (1 E).

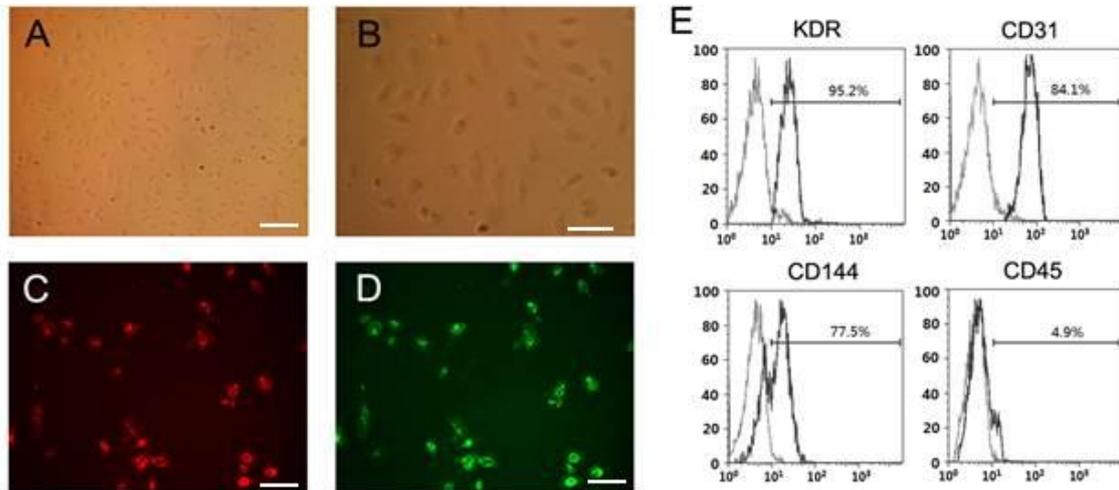


Figure 1. Identification of EPCs by flow cytometry and confocal laser scanning microscope $\times 200$, Scale bars = 20 μm (n=3).

Effect of FGF2 on apoptosis of EPC cells exposed to high glucose conditions

Based on TUNEL assays, when EPCs were exposed to high glucose conditions for 48 hours, apoptosis rates were significantly higher than in the normal control group (** $p < 0.01$). The levels of apoptosis in EPCs were reduced significantly after exogenous FGF-2 treatment. 150 ng/mL FGF-2 treatment had the greatest decline in apoptosis after high glucose exposure (Figure 2, # $p < 0.05$, ## $p < 0.01$).

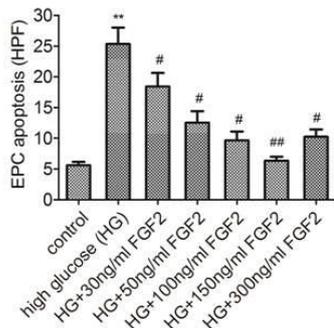


Figure 2. Apoptosis rate of EPCs after high glucose exposure with/without FGF2 (n=3).

Effect of FGF2 on migration rates of EPC cells exposed to high glucose conditions

Transwell chamber assays demonstrated that the migration of EPCs in the high glucose group were significantly lower than the control group (** $p < 0.01$). Compared with the high glucose group, the addition of FGF-2 increased the number of cell migration. A concentration of 150 ng/mL FGF-2 had the most significant increase in cell migration compared to the high glucose treatment group (Figure 3, # $p < 0.05$, ## $p < 0.01$).

Effect of FGF2 on EPC's tube formation ability under high glucose conditions

Tube formation assays showed that in the high glucose group, the EPCs' lumen number was significantly lower than in the normal group (** $p < 0.01$). Exogenous FGF-2 treatment significantly enhanced tube formation ability, and with the increase of FGF-2 concentrations, the lumen number increased accordingly. At 150 ng/mL FGF-2, tube formation ability achieved the highest levels (Fig. 4, # $p < 0.05$, ## $p < 0.01$).

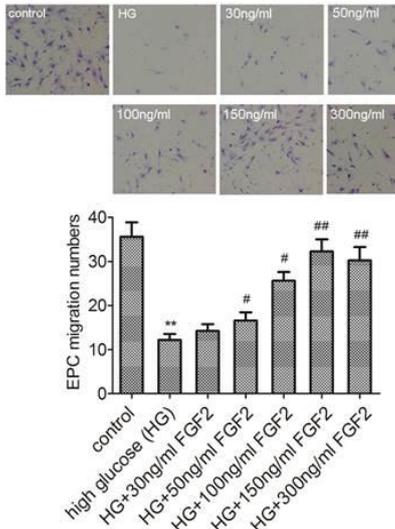


Figure 3. EPC cell migration after high glucose exposure and treatment with/without FGF-2 in Transwell chambers×200(n=3)

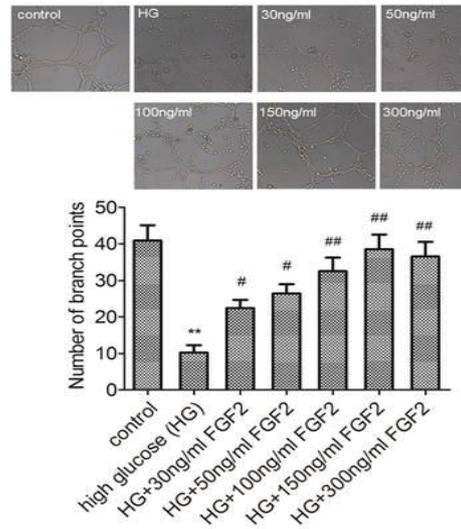


Figure 4. Tube formation of EPCs in Matrigel×200. Cells were exposed to high glucose concentrations with/without FGF-2 treatment (n=3)

Effect of FGF2 on the Wnt/ β -catenin's signaling pathway under high glucose conditions

Western blot analysis demonstrated that the activity ratio of EPC's β -catenin in high glucose group was significantly lower than in the control group (* $p < 0.05$).

The addition of exogenous FGF-2 significantly increased the activity of β -catenin, and increased with increasing concentrations of FGF-2. The activity of β -catenin was highest at 150 ng/mL of FGF-2 treatment (Figure 5, # $p < 0.05$, ## $p < 0.01$).

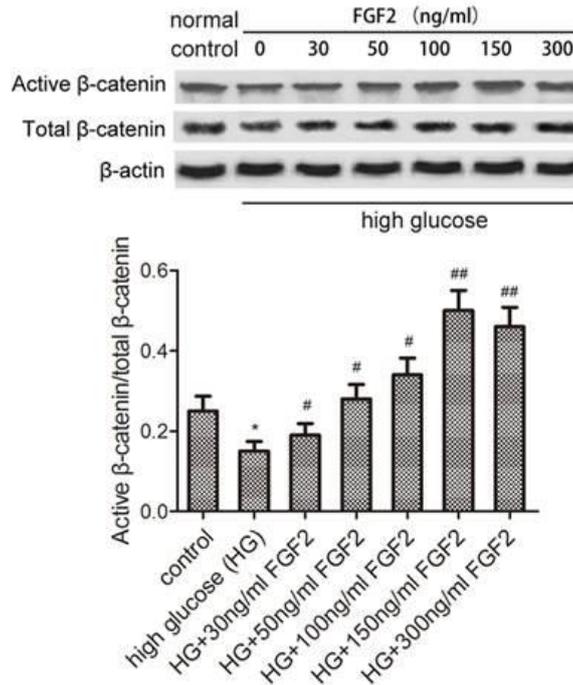


Figure 5. The ratio of active/total Wnt/ β -catenin in EPCs exposed to high glucose with/without FGF-2 treatment (n=3).

Effect of FGF2 on the function of EPCs exposed to high glucose conditions

Laser Doppler flow cytometry measurements showed that after 4 weeks of injecting normal EPCs, mice had a significant increase in blood flow to the

ischemic hind limbs, while injection of EPCs cultured in high glucose showed an obvious decrease. Injection of EPCs cultured in high glucose and FGF-2 showed an obvious increase to that of EPCs cultured in high glucose only (Figure 5).

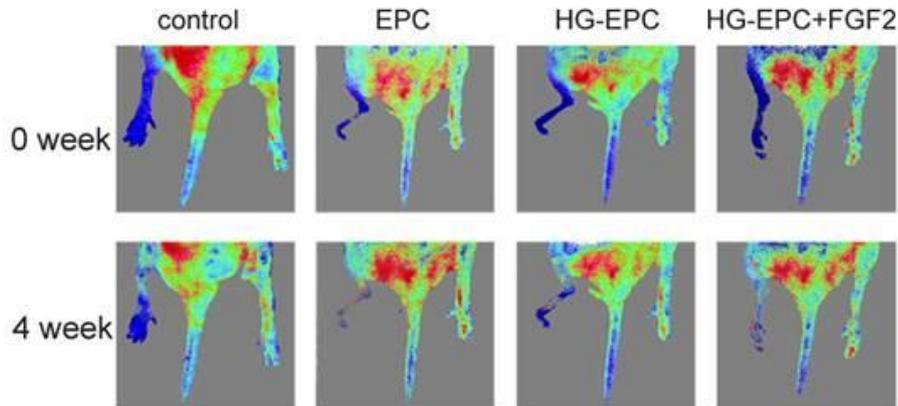


Figure 6. Mouse ischemic hind limb blood flow measured using Laser Doppler Flowmetry (n=6).

DISCUSSION

The impaired function and senescence of EPCs in hyperglycemic conditions contributes to increased ROS and impedes the healing of diabetic foot ulcers (DFUs), which are largely caused by peripheral neuropathy associated with diabetes mellitus (DM) (3, 4). Microcirculatory disturbance is an important pathological basis for DFUs, which is formed under the combined effects of vascular endothelial dysfunction and autonomic nerve regulation dysfunction in diabetic patients, resulting in tissue ischemia (5). Factors released by ischemic tissue can mobilize and recruit stem precursor cells to the site of injury to replace and repair damaged tissues (6).

Angiogenesis is a multistage process involving multiple growth factors, cytokines, and the cell involvement (7). EPCs have the ability to proliferate, adhere, migrate, and form specific vascular structures (8). EPCs can be used to repair damaged blood vessels (9). EPCs transplantation has been widely applied to promote angiogenesis of ischemic tissue (10). Autologous EPCs transplantation has many advantages including pain relief and ease of collection (11). However, the advantage of using autologous EPCs transplantation was not observed to be very efficacious in patients with diabetes, because high glucose impaired the function of the EPCs.

EPCs, which express specific cell surface markers, CD34⁺ and KDR⁺, can be isolated from peripheral blood. When transplanted into ischemia animal models, it can integrate into new capillaries at the tissue level, and can enhance the growth of collateral vessels in ischemic tissues (12, 13). EPCs express two surface markers, CD34 and CD133, and endothelial cell surface marker VEGF-R2 (also known as KDR) (14). During the initial stages of differentiation, EPCs proliferate rapidly and have high angiogenesis ability, but the proliferation rates decrease as they differentiate into myeloid cells or endothelial cells (15-17). As EPCs differentiate, the expression of certain cell surface receptors decrease, i.e. late EPCs hardly express CD133, KDR and CD34 (18), however all of them start to express CD31, CD144, CD146, CD105, vWF and Enos. Flow

cytometry analysis revealed that the isolated EPCs used in our study express surface antigen markers KDR, CD31 and CD144 associated with endothelial progenitor cells, but do not express the hematopoietic stem cell's surface antigen marker CD45. After seven days' of culture, spindle adherent cells spring out from the edges of the cells mass, and start to express FITC-UEA-I and Dil-ac-LDL, which are markers for endothelial cells.

FGF-2 can induce the secretion of many growth factors including VEGF, HGF, MCP-1, IL-1, beta and IL-8, however the levels of FGF-2 is low in EPCs. In addition, high glucose levels would result in abnormal paracrine and autocrine signaling in FGF-2 production in EPCs. Studies have demonstrated that in DM patients, EPCs have lower secretion of stem cell factors, growth factors, thrombopoietin, and increased levels of IL-1 β , and TNF- α . High glucose levels also induce oxidative stress, resulting in EPC dysfunction and apoptosis in diabetic patients (19, 20). Hyperglycemia also decreases EPC activity, migration, and tube formation (21). Therefore, targeting oxidative stress may be important for preventing impaired vascular homeostasis in DM.

In our study, in vitro conditions of high glucose resulted in increased levels of EPC apoptosis and decreased migration and tube formation, indicating high glucose levels led to the dysfunction of EPC. FGF-2 plays an important role in angiogenesis by stimulating synthesis of metalloproteinases and urokinase type plasminogen activators, which leads to basement membrane degradation, cell migration and formation of new blood vessels (22). FGF-2 also plays a role in the synthesis of extracellular matrix, the promotion and maturation of new blood vessels (23), and the synthesis of vascular endothelial growth factor VEGF (24). In addition, FGF-2 plays an important role in proliferation, differentiation, angiogenesis, wound healing, embryonic development and differentiation of multiple organs. Recent studies have demonstrated that high levels of FGF-2 can increase levels of EPCs' white cell mediators (such as IL-8), accompanied by down regulation of eotaxin. However, the role of FGF-2 in reversing EPC dysfunction during hyperglycemia has

not been studied. We investigated the effect of exogenous FGF-2 on the reversal of EPC dysfunction. We found that FGF-2 decreased the levels of apoptosis observed in EPCs exposed to high glucose, while simultaneously increasing the migration and tube formation ability of EPCs. In vivo, high glucose interfered with the ability of FGF-2 to reverse ischemia. Wnt signaling is known to be a critical factor in modulating angiogenesis and regulating the mobilization of EPCs. Studies have demonstrated that Wnt/ β -catenin signaling pathway plays a key role in vascular biology and activation of the Wnt signaling pathway to promote vascular regeneration after limb ischemia (25). Whether FGF-2 relies on the Wnt/ β -catenin signaling pathway to regulate its function in EPCs has not been demonstrated. However, we have demonstrated that exogenous treatment with FGF-2 results in activation of the

Wnt/ β -catenin pathway, which indicates that Wnt/ β -catenin activators could promote the functional recovery of dysfunctional EPCs induced by hyperglycemia.

Based on our results, we hypothesize the following: In autologous EPC transplantation, better efficacy could be achieved for DM patients, with exogenous FGF-2 treatment. FGF-2 could improve the therapeutic effects of autologous cell transplantation by reversing EPC dysfunction and enhancing angiogenesis. However, a standard phenotype and functional analysis for EPCs has not been established for clinical use, and there are still many controversies in EPC biology that needs to be clarified. Further research into EPC biology and transplantation procedures needs to be performed before it can be taken into the clinic for the treatment of DFUs.

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