Abstract. Myocardial infarction is a major contributor to morbidity and mortality in diabetes, which is characterized by inadequate angiogenesis and consequent poor blood reperfusion in the diabetic ischemic heart. The aim of the present study was to investigate the effect that oxidative stress in endothelial progenitor cells (EPCs) has on cardiac angiogenesis in diabetic mice. EPCs derived from diabetic mice revealed reductions in superoxide dismutase (SOD) expression levels and activity compared with those from normal mice. An endothelial tube formation assay showed that angiogenesis was markedly delayed for diabetic EPCs, compared with normal controls. EPCs subjected to various pretreatments were tested as a cell therapy in a diabetic mouse model of myocardial infarction. Induction of oxidative stress in normal EPCs by H$_2$O$_2$ or small interfering RNA-mediated knockdown of SOD reduced their angiogenic activity in the ischemic myocardium of the diabetic mice. Conversely, cell therapy using EPCs from diabetic mice following SOD gene overexpression or treatment with the antioxidant Tempol normalized their ability to promote angiogenesis. These results indicate that decreased expression levels of SOD in EPCs contribute to impaired angiogenesis. In addition, normalization of diabetic EPCs by ex vivo SOD gene therapy accelerates the ability of the EPCs to promote angiogenesis and improve cardiac function when used as a cell therapy following myocardial infarction in diabetic mice.

Introduction

In diabetes, hyperglycemia is a major cause of the production of reactive oxygen species (ROS) and oxidative stress (1,2). Hyperglycemia also activates nicotinamide adenine dinucleotide phosphate oxidase or reduces guanosine triphosphate cyclohydrolase 1 activity, resulting in superoxide anion generation in vascular endothelial cells or tissue (3,4). ROS production in diabetes leads to cellular damage and limited neovascularization, such as angiogenesis in the ischemic heart (5,6).

As a major cell component involved in angiogenesis, endothelial progenitor cells (EPCs) migrate to sites of injury and contribute to angiogenesis in the process of vascular maintenance in the ischemia heart (7). Numerous studies have found that reductions of circulating EPC numbers and function are critical in wound repair in diabetic patients (8-11). However, the mechanisms underlying EPC dysfunction in diabetes require further elucidation.

High glucose levels or hyperglycemia induce oxidative stress and damage EPC survival by reducing their migration or proliferation (12,13). As a feed-back regulator, the levels of the antioxidant enzyme superoxide dismutase (SOD) in normal EPCs are high and important in the resistance of EPCs to oxidative stress (14,15). A loss of resistance of EPCs to the oxidative stress induced by high glucose levels may mediate EPC dysfunction in diabetes. Therefore, the present study investigated the hypothesis that increased oxidative stress in EPCs may be crucial to delayed angiogenesis in the ischemic heart of diabetes.

Materials and methods

Materials. Monoclonal antibodies targeting the following: Rabbit polyclonal IgG manganese superoxide dismutase (Mn-SOD; cat no. sc-30080); mouse monoclonal IgG cluster of differentiation (CD)31; cat no. sc-71873) and mouse monoclonal IgM glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cat no. sc-59540) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology, Inc. All concentrations mentioned are final concentrations, following dilution with buffer.
Mouse model of diabetes. The db/db mice (weight, 30-40 g) and control mice (weight, 20-25 g) were used to investigate angiogenesis, as described previously (16). There were 15-20 mice in each group, and all were housed in temperature-controlled cages with 12-h dark:light cycle and free access to food and water. In brief, the mice used in the present study were 10-14-week-old adult male diabetic (db/db) and non-diabetic healthy heterozygotes (db/+). purchased from the Jackson Laboratory through Shanghai Maosheng Biologic Science & Technology Development Co., Ltd., (Shanghai, China). All animal procedures were performed with approval of the Harbin Medical University Animal Care and Use Committee (Harbin, China).

Isolation and characterization of EPCs from peripheral blood. The isolation, ex vivo expansion and culture of EPCs was performed as previously described (17). In brief, under anesthesia, peripheral blood samples were collected from the mice and mononuclear cells were isolated and purified from the blood. Freshly isolated mononuclear cells from peripheral blood were characterized using flow cytometry, as previously described (18). EPCs were identified by labeling with Dil-acetylated low density lipoprotein (Dil-ac-LDL; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin (Sigma-Aldrich, St. Louis, MO, USA). In order to mimic the *in vivo* environment of EPCs, EPCs from diabetic mice were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; 30 mM D-glucose; cat no. 11965-126; Thermo Fisher Scientific, Inc.). EPCs from control mice were cultured in normal glucose DMEM (5 mM D-glucose; cat no. 10567-014; Thermo Fisher Scientific, Inc).

Measurement of ROS levels in EPCs. The levels of ROS in the EPCs from normal and diabetic mice were measured using a previously described dihydroethidium (DHE) fluorescence/high-performance liquid chromatography (HPLC) assay with minor modifications (19). Briefly, EPCs were incubated with DHE (10 µM; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min, homogenized, and subjected to methanol extraction. HPLC was performed using a C-18 column (cat no. 077974; Thermo Fisher Scientific, Inc.) with a mobile phase comprising a gradient of acetonitrile and 0.1% trifluoroacetic acid, to separate and quantify oxethidium (ROS oxidation product of DHE) and ethidium (product of DHE auto-oxidation). ROS production was determined by conversion of DHE into oxyethidium.

Western blot analysis. Cell lysates were subjected to western blot analysis, as described previously (4). EPCs were lysed using CelLytic MT lysis buffer (Sigma-Aldrich) with protease inhibitor cocktail (100 µl protease inhibitor/10 ml lysis buffer; Sigma-Aldrich). The protein content was assayed by bicinchoninic acid assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Proteins (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was incubated with a 1:1,000 dilution of primary antibody targeting Mn-SOD or GAPDH, followed by a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by electrochemiluminescence (ECL Prime Western Blotting Detection Reagent; cat no. RPN2232; GE Healthcare Life Sciences, Shanghai, China). The intensity (area x density) of the individual bands on the western blots was measured by densitometry (model GS-700 Imaging Densitometer; Bio-Rad Laboratories, Inc.). The background was subtracted from the calculated intensity.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The protocol of the RT-qPCR was as described previously (20). In brief, EPCs were lysed and RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For each sample, 2 µg RNA was reverse transcribed into cDNA using SuperScript II RT, Oligo dT (both Invitrogen; Thermo Fisher Scientific, Inc.) and dNTP mix (Promega Corporation, Madison, WI, USA), with a GeneAmp PCR 9700 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was conducted using SYBR Green PCR Master mix with a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: Mn-SOD forward, 5’-CACATTAACCGCGAGATC ATG-3’, and reverse 5’-CCAGAGCTGCTGTTACCTTCTC-3’; and GAPDH forward, 5’-ATGGGTGGTGACCAGCCA-3’, and reverse 5’-CGGCCCCGAGCTTTTTGTG-3’. Data was normalized according to the 2^(-ΔΔCq) method (20).

Adenovirus infection of EPCs. As described previously (21), EPCs from diabetic mice were infected with adenoviral vector pShuttle cytomegalovirus containing Mn-SOD (Ad-Mn-SOD) or green fluorescent protein (GFP) marker gene (Ad-GFP) overnight in plasmid transfection medium (cat no. sc-108062; Santa Cruz Biotechnology, Inc.) supplemented with 2% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were then washed and incubated in fresh medium for an additional 12 h prior to experimentation. These conditions typically produced an infection efficiency of ≥80%, as determined by measurement of GFP expression.

Transfection of siRNA in EPCs. After 7 days of culture, EPCs from normal mice were transfected with Mn-SOD siRNA in 6-well plates following a previously described protocol (22).

Tube formation. Tube formation was investigated as described previously (23). Cultured EPCs were seeded on cell culture dishes coated with growth factor-reduced Matrigel (BD Biosciences) and cultured in MCDB 133 (Gibco) medium containing 0.5% fetal bovine serum. After 24 h, the medium was removed and the cells were fixed with 4% paraformaldehyde. Photographic images were captured through a microscope (model no. IX83; Olympus, Tokyo, Japan). The capillary tube area was quantified per µm² using ImageJ image analysis software (National Institutes of Health, Bethesda, MD, USA).

Migration assay. The migration of EPCs toward a gradient of stromal cell-derived factor-1 (SDF-1) was performed using a CytoSelect 24-Well Cell Migration Assay kit (cat no. CBA-107, Cell Biolabs, Inc., San Diego, CA, USA) in a 24-well Transwell chamber (8.0 µm pore size, polycarbonate membrane; Corning Incorporated, Corning, NY, USA). Briefly, chemotaxis buffer (serum-free EBM-2, 0.1% BSA) was added to the lower
Echocardiography. Echocardiography was performed as described previously (26). Ultrasonography with standard parasternal and apical views was performed in mice in the left lateral recumbent position. High-quality 2-dimensional images including apical (4-chamber, 2-chamber and long-axis) and short-axis views (mitral annulus, papillary muscle and apex) were obtained with the use of a 2.0-4.0 MHz transducer.

Capillary density. Histological analysis was performed on perfusion-fixed hearts collected from mice at 28 days after surgery (n=5-6 mice/group). Briefly, the mice were euthanized by pentobarbital (50 mg/kg). The chest was opened and the heart was arrested in diastole by intraventricular injection of KCl (10%). The right atrium was then cut and the myocardial vasculature was perfused, followed by 10 min perfusion with 10% formalin. The hearts were harvested and fixed in 4% formalin for 24 h. The formalin-fixed tissues were embedded in paraffin wax and cut into 5 µm sections. For the measurement of capillary density (counts/mm²), immunohistochemical analysis of CD31 (also known as platelet endothelial cell adhesion molecule-1; 1:50; Santa Cruz Biotechnology, Inc.) was performed. Transverse sections of the short axis of the left ventricle for each sample were used in this analysis. Five fields on the slide were randomly selected for counting the stained capillaries in the border zone between the infarcted area and non-infarcted area at x400 magnification. All stained capillaries were counted, and the density was expressed as number/mm².

Statistical analysis. Quantitative data are presented as the mean ± standard error of the mean. All statistical analyses were performed using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). Figures were generated using GraphPad Prism software (version 5.1; GraphPad Prism Software Inc., San Diego, CA, USA). For multiple comparisons among ≥3 groups, one-way analysis of variance followed by Tukey post-hoc tests or Bonferroni corrections were used. A two-sided P-value <0.05 was considered as significant for all statistical procedures used.

Results

Hyperglycemia induces oxidative stress in EPCs in vivo. The present study investigated whether oxidative stress in EPCs was increased in diabetic mice. Adult male diabetic mice (db/db) exhibited hyperglycemia (314.52±39.18 mg/dl) compared with
normal control db/+ mice (179.08 ± 26.38 mg/dl). As shown in Fig. 1A, the level of ROS was significantly increased in EPCs from diabetic mice, compared with that in EPCs from control mice. These data suggest that oxidative stress may be an important factor contributing to the dysfunction of EPCs in diabetic mice.

SOD is reduced in EPCs derived from diabetic mice. Mn-SOD protect the functions of EPCs against oxidative stress (27). Thus, whether SOD levels were decreased in EPCs from diabetic mice was investigated. The mRNA levels of Mn-SOD were examined using RT-qPCR. As shown in Fig. 1B, Mn-SOD mRNA expression levels were significantly reduced in the EPCs from diabetic mice, compared with those in control mice. Consistent with RT-qPCR results, the levels of Mn-SOD protein (Fig. 1C) and Mn-SOD activity (Fig. 1D) were also suppressed in EPCs from diabetic mice.

Hyperglycemia impairs the tube formation and migration functions of cultured EPCs. To investigate EPC functions, the production of growth factors by EPCs was assayed. As shown in Fig. 2A, the levels of the growth factors vascular endothelial growth factor and basic fibroblast growth factor in the culture medium, were decreased in EPCs derived from diabetic mice, indicating that oxidative stress caused by hyperglycemia may reduce growth factor production and secretion.

Tube formation of EPCs on Matrigel was then investigated. As shown in Fig. 2B, diabetic EPCs formed fewer networks than normal EPCs. A modified Transwell chamber migration assay was performed to investigate the migration of EPCs from normal and control mice. As shown in Fig. 2C, diabetic EPCs showed significantly lower migration in response to SDF-1, as compared with control EPCs. Collectively, these data suggest that hyperglycemia impaired the functions of tube formation and migration in cultured EPCs.
Oxidative stress-mediated dysfunction of EPCs from diabetic mice. To further investigate whether diabetes damaged the functions of EPCs via oxidative stress, an antioxidant was used or \textit{ex vivo} Mn-SOD gene therapy was performed to suppress oxidative stress in EPCs from diabetic mice. Tube formation of EPCs on Matrigel was assayed. As shown in Fig. 3, treatment with Tempol antioxidant or overexpression of Mn-SOD increased the ability of diabetic EPCs to form tube networks on Matrigel compared with unmodified diabetic EPCs.

Angiogenesis is a central feature of recovery in ischemic hearts and depends on cell proliferation, migration, and capillary tube formation in EPCs (28). Oxidative stress in diabetic EPCs was examined to determine whether it contributed to delayed angiogenesis following myocardial infarction. Control EPCs were treated with H$_2$O$_2$ oxidant (Fig. 4A) or transfected with Mn-SOD siRNA to induce the silencing of Mn-SOD expression in EPCs (Fig. 4B). Low doses of H$_2$O$_2$ and Mn-SOD siRNA did not alter cell viability, as determined by MTT assay (data not shown), although Mn-SOD siRNA specifically reduced Mn-SOD protein expression (Fig. 4B). These conditioned EPCs were transplanted by tail-vein
Oxidative stress attenuates EPC-induced cardiac functional recovery following myocardial infarction in diabetic mice. Whether oxidative stress-impaired angiogenesis contributed to delayed recovery of cardiac functional recovery following myocardial infarction in diabetic mice was investigated. Left ventricular functional parameters were examined by echocardiography 30 days after surgery. As shown in Fig. 5, left ventricular function was diminished in mice injected with \( \text{H}_2\text{O}_2 \)-treated (Fig. 5A) or Mn-SOD siRNA-treated EPCs. These results demonstrated that the induction of oxidative stress in normal EPCs delayed neovascularization by increasing capillary number following myocardial infarction.

Suppression of oxidative stress in EPCs improves heart function and promotes angiogenesis in the diabetic hearts of mice following myocardial infarction. The aforementioned data demonstrate that EPCs isolated from diabetic mice exhibited decreased Mn-SOD levels and elevated oxidative stress, and that gene therapy of diabetic EPCs improved their ex vivo tube formation ability. To determine whether the suppression of oxidative stress has beneficial effects on angiogenesis and promotes heart function, upregulation of the anti-oxidative system was performed by treatment with Tempol (Fig. 6A) or Mn-SOD gene therapy (Fig. 6B) in diabetic EPCs prior to transplantation. EPCs from diabetic mice pre-treated with Tempol or infected with Ad-Mn-SOD exhibited improved cardiac function and increased angiogenesis, respectively, in the ischemic heart. These data suggest that Mn-SOD may be important for normal EPC function to promote the recovery of the ischemic heart in diabetes.

**Discussion**

The present study demonstrated that hyperglycemia caused oxidative stress in EPCs, resulting in the dysfunction of EPCs, which in turn attenuated repair in the ischemic heart. Furthermore, suppression of oxidative stress in EPCs from diabetic mice rescued their ability to improve cardiac function following myocardial infarction. The mechanism underlying this process may be due to decreased SOD mRNA and protein expression levels, leading to a reduction in EPC resistance to oxidative stress. These results suggest that deficiency of SOD is a key factor for EPC dysfunction and contributes to delayed recovery of the ischemic heart in diabetes.

The results of the present study demonstrated that normalization of diabetic EPCs by suppression of oxidative stress accelerated angiogenesis and improved cardiac function in diabetic mice following myocardial infarction. Myocardial infarction, which is predominantly caused by atherosclerosis, is a severe threat to human health. Following myocardial infarction, abnormal myocardial blood flow may impair myocardial \( \text{O}_2 \) delivery, resulting in maladaptive remodeling of the left ventricle, including cardiac dysfunction, apoptosis and fibrosis (29,30). Numerous compensatory responses of the body occur following myocardial infarction, including spontaneous neovascularization, but this is not sufficient for the heart to fully recover (31,32). However, the process of angiogenesis is markedly delayed in patients with diabetes.
due to the dysfunction of EPCs (33-35). Therefore, therapeutic angiogenesis is a potentially useful approach for the treatment of ischemic cardiovascular disease in diabetes (36-38). Marrotte et al (16) reported that normalization of diabetic EPCs by inhibition of oxidative stress improved wound healing in diabetic mice, results that are concordant with those of the present study.

In myocardial infarction, vasculogenesis is the process of blood vessel formation, during which EPCs migrate and fuse with other EPCs and differentiate into endothelial cells while forming new blood vessels (39). Primary endothelial cells escape from their original location by crossing through the basement membrane and migrating toward an angiogenic stimulus. The transported endothelial cells subsequently proliferate to enable the formation of a new blood vessel (40). Following this proliferation, the new outgrowth of endothelial cells reorganizes to form a unique three-dimensional open tubular structure. Therefore, both endothelial cells and EPCs contribute to revascularization. Hyperglycemia is also a risk factor for the development of endothelial dysfunction in diabetic patients (4). Therefore, EPCs are not the only factor inducing ischemic angiogenesis. The role of endothelial dysfunction in diabetes-delayed angiogenesis requires further investigation.

In summary, the results of the present study indicate that cell therapy using diabetic EPCs normalized by ex vivo gene therapy with SOD is a potentially useful approach for accelerating the ability of EPCs to promote angiogenesis in the treatment of ischemic diseases, such as diabetic foot, vascular stiffness, myocardial infarction and wound repair in diabetes. Future studies are required to elucidate the molecular mechanism concerning how diabetes is able to reduce SOD activity in EPC.

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