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Oxidative stress tolerance of early stage diabetic endothelial progenitor cell

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ABSTRACT

Introduction: One of the causes for poor vasculogenesis of diabetes mellitus (DM) is known to rise from the dysfunction of bone marrow-derived endothelial progenitor cells (BM EPCs). However, the origin of its cause is less understood. We aimed to investigate the effect of oxidative stress in early stage of diabetic BM-EPC and whether its vasculogenic dysfunction is caused by oxidative stress.
Methods: Bone marrow c-Kit+Sca-1+Lin− (BM-KSL) cells were sorted from control and streptozotocin-induced diabetic C57BL6J mice by flow cytometry. BM-KSLs were then assessed for vasculogenic potential (colony forming assay; EPC-CFA), accumulation of intracellular ROS (CM-H2DCFDA), carbonylated protein (ELISA), anti-oxidative enzymes expression (RT-qPCR) and catalase activity (Amplex Red).
Results: Compared to control, DM BM-KSL had significantly lower EPC-CFUs in both definitive EPC-CFU and total EPC-CFU ($p < 0.05$). Interestingly, the oxidative stress level of DM BM-KSL was comparable and was not significantly different to control followed by increased in anti-oxidative enzymes expression and catalase activity.
Conclusions: Primitive BM-EPCs showed vasculogenic dysfunction in early diabetes. However the oxidative stress is not denoted as the major initiating factor of its cause. Our results suggest that primitive BM-KSL cell has the ability to compensate oxidative stress levels in early diabetes by increasing the expression of anti-oxidative enzymes.

1. Introduction

Dysfunction of endothelial progenitor cell (EPC) is thought to be the underlying mechanism of neovascularization impairment and vascular complications in diabetes. EPC was first described as a specialized subset of hematopoietic progenitor cells, reside in bone marrow as a pool, circulate in peripheral blood and facilitate neovascularization in physiological and pathological conditions [1,2].

Abbreviations: BM-KSL, bone marrow derived-c-Kit+Sca-1+Lin−; BM-EPC, bone marrow-derived endothelial progenitor cells; DM BM-KSL, diabetic BM-KSL; EPC-CFA, endothelial progenitor cell colony forming assay; EPC-CFU, endothelial progenitor cell colony forming unit; QQc, quality and quantity culture system; pEPC-CFU, primitive/small EPC-CFU; dEPC-CFU, definitive/large EPC-CFU; tEPC-CFU, total EPC-CFU.

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Consistent data have supported that EPC is dysfunctional in diabetes, as represented by impairment in proliferation [3–5], migration/mobilization [6–8], colony forming capacity [7–9], and tubular formation [6].

Oxidative stress is thought as one of the factors responsible for impairment of neovascularization and complications in diabetes [10–12]. Reactive oxygen species (ROS) induced in diabetes down regulates HIF-1α and weakening of SDF-1α and VEGF signaling which disturb cells mobilization into injury site [13,14]. ROS also thought to be responsible for EPC reduction in diabetes through ROS-mediated apoptosis [15]. Nevertheless, previous studies on the effect of oxidative stress on EPC dysfunction are performed on differentiated EPC from cultured of peripheral blood (PB) or BM mononuclear cells. The study of oxidative stress on early stage diabetic EPCs from BM hematopoietic population is not yet elucidated.

Recently Masuda et al., developed an EPC-colony forming assay (EPC-CFA) to distinguish vasculogenic function of primitive or early stage EPCs from hematopoietic stem cell population such as c-Kit+/Sca-1+/Lin− (KSL) cells in mice and CD34+ or CD133+ cells in human. EPC colony forming unit (EPC-CFU) consists of two different EPC colonies: small (primitive; pEPC-CFU) colonies consist of highly immature and proliferative population of EPCs and large (definitive; dEPC-CFU) with high differentiation potential in ready state for vasculogenesis [7,16,17].

Using EPC-CFU, our group reported vasculogenic impairment of early stage diabetic BM-KSL (DM BM-KSL) by decreased number of definitive and total EPC-CFU [7,9], however the mechanism or the direct cause is not yet elucidated. Since previous reports show that oxidative stress is one of the causes of vasculogenic impairment in cultured EPCs, herein we will explore the effect of oxidative stress on vasculogenic potential of diabetic BM vascular progenitor cells from KSL cell population. Using QQc system as facilitating tool, we aim to investigate whether improvement in diabetic EPC post-QQc will follow by decrease in oxidative stress level. To our knowledge, this is the first study to investigate whether diabetic vasculogenic dysfunction resides on the oxidative stress damage of primitive bone marrow EPCs.

2. Materials and methods

2.1. Animals and STZ-induced diabetic mouse model

All the experimental protocols described were approved by the Animal Care Committees of Juntendo University. We used male 8 weeks old C57BL/6J (20–25 g) mice (Sankyo Laboratory) which were housed in specific pathogen-free barrier facilities and maintained under a 12-hour light/dark cycle. Diabetes (DM) were induced by intraperitoneal injection of Streptozotocin (STZ, 50 mg/kg, Sigma) dissolved in citrate buffer (pH 4.5) for 5 consecutive days, as previously described [9,18]. Mice with blood glucose level equal or higher than 300 mg/dl were decided as diabetic mice which maintained at least for 4 weeks.

2.2. Bone marrow (BM) derived KSL cells isolation

BM cells were harvested from femur, tibia, pelvis and humerus bones of DM and control mice as previously described [9]. Erythrocytes were removed by ammonium chloride. The cells were then labeled by biotin-conjugated antibody cocktail (CD3e, CD45/B220, Ly-6G and Ly-6C, CD-11b, TER-119) (all antibodies obtained from eBioscience) and followed by anti-biotin micro-beads depletion by AutoMACS (Militenyi) to obtain lineage negative (Lin−) cells. The Lin− cells were stained with APC-labeled Sca-1 and PE-labeled c-Kit antibodies (eBioscience), and sorted for c-Kit+Sca-1+Lin− (KSL) cells by FACS Aria (Becton Dickinson).

2.3. Serum free quantity and quality culture (QQc) system

Previously our group established a serum-free quality and quantity culture (QQc) system containing thrombopoietin (TPO), VEGF, Stem Cell Factor (SCF), IL-6 and Flt-3 ligand as an optimal quality and quantity culture for EPC expansion to enhance their vasculogenic and regenerative potential [19]. BM-KSL of 1×10^3 cells were cultured in QQc system as previously described [9,19,20]. Seven days later cells were harvested and the collected cells were grouped accordingly as DM post-QQc for DM BM-KSL cells.

2.4. EPC colony formation assay (EPC-CFA)

EPC vasculogenic CFA was performed and characterized as previously described [7,9,16,17,20]. Briefly a total of 500 BM-KSL cells/dish suspends in semi-solid culture medium for EPC-CFA and were seeded into 35 mm primaria culture dish. The frequency of primitive/small (pEPC-CFU), definitive/large (dEPC-CFU) and total colony forming unit (tEPC-CFU) were assessed on day-7 by two investigators who were blinded to the experimental conditions. Experiments were performed in triplicate.

2.5. Intracellular ROS detection

Intracellular ROS levels were evaluated with 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) by followed the manufacture's protocol. Freshly prepared BM-KSL and post-QQc BM-KSL cells from control and or diabetic mice were labeled with CM-H2DCFDA and incubated for 15 min at 37 °C. The CM-H_2DCFDA labeled cells were then analyzed using FACS Calibur (Becton Dickinson).

2.6. Protein carbonyl detection for oxidative stress marker

Protein was extracted with RIPA buffer (Thermo Scientific) with protease inhibitor cocktail (Roche). Protein was derivatized with DNPH (2,4-dinitrophenyl hydrazine) as previously described [21]. After removing the excess of DNPH, 96-well ELISA plates were coated at 4 °C overnight with samples. After removing them, the wells were blocked with 3%BSA/PBS at 37 °C for 1 h. Anti DNPH antibody (1:150, Millipore) was added and incubated at 37 °C for 1 h. HRP-labeled anti-rabbit antibody (1:300, Millipore) was added and incubate at 37 °C for 1 h (both first and secondary antibodies were part of Oxyblot Kit, Millipore, Chemicon International). To develop color the o-phenylendiamin dihydrochloride (Sigma) was added for 40 min and stopped by 1 N sulfuric acid. The absorbance was measured at 492 nm [22]. The result presents as ratio of carbonylated protein.

2.7. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. cDNA was synthesized using SuperScript First-Strand Synthesis System (Invitrogen). The genes expression was measured on an ABI 7500 FAST Real-Time PCR system using TaqMan probes 18S rRNA (ribosomal RNA control reagents), MnSOD (Sod2, Mm01313000_m1), catalase (Cat, Mm00437992_m1) and glutathione Peroxidase-1 (Gpx1, Mm00656767_g1) (Life Technologies). Relative expression of the target gene was demonstrated by the ΔΔCt.

2.8. Catalase activity

BM-KSL cells (as minimal 2×10^4 cells) were extracted in 0.2% Triton X-100 in 0.1 M phosphate buffer (pH 7.7) and extracts were