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Diabetes & Metabolism 43 (2017) 154–162

Original article

Impaired development and dysfunction of endothelial progenitor cells in type 2 diabetic mice

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Received 26 January 2016; received in revised form 2 July 2016; accepted 23 July 2016
Available online 13 September 2016

Abstract

Aim. – Dysfunction of circulating endothelial progenitor cells (EPCs) has been shown to affect the development of microvascular diseases in diabetes patients. The aim of this study was to elucidate the development and mechanical dysfunction of EPCs in type 2 diabetes (T2D).

Methods. – The colony-forming capacity of EPCs and differentiation potential of bone marrow (BM) c-Kit(+)/Sca-I(+) lineage-negative mononuclear cells (KSL) were examined in T2D mice, *db/db* mice and KKA^{y} mice, using EPC colony-forming assay (EPC-CFA).

Results. – T2D mice had fewer BM stem/progenitor cells, and proliferation of KSL was lowest in the BM of *db/db* mice. In T2D mice, the frequency of large colony-forming units (CFUs) derived from BM-KSL was highly reduced, indicating dysfunction of differentiation into mature EPCs. Only a small number of BM-derived progenitors [CD34(+) KSL cells], which contribute to the supply of EPCs for postnatal neovascularization, was also found. Furthermore, in terms of their plasticity to transdifferentiate into various cell types, BM-KSL exhibited a greater potential to differentiate into granulocyte macrophages (GMs) than into other cell types.

Conclusion. – T2D affected EPC colony formation and differentiation of stem cells to mature EPCs or haematopoietic cells. These data suggest opposing regulatory mechanisms for differentiation into mature EPCs and GMs in T2D mice.

Keywords: Colony-forming assay; Diabetes; Differentiation; Endothelial progenitor cells; Granulocyte macrophages

1. Introduction

Endothelial progenitor cell (EPC) dysfunction is involved in a variety of vascular diseases [1–6]. Recently, various studies have shown that EPC dysfunction in patients at risk of cardiovascular disease contributes to the development of atherosclerosis and ischaemic vascular diseases [7,8]. In particular, patients with type 2 diabetes (T2D) are at increased risk of atherosclerotic disease and poor outcomes after vascular occlusion, and also have a large number of associated complications, such as microvascular diseases [9–11]. Several studies have shown a negative correlation between the number of EPCs and HbA_{1c} levels in T2D patients, as well as the role of EPC dysfunction in adhesion, proliferation and tubular formation in diabetes [12,13].

Abbreviations: EPCs, endothelial progenitor cells; EPC-CFA, EPC colony-forming assay; CFU, colony-forming unit; PB, peripheral blood; BM, bone marrow; MNC, mononuclear cell; LN-, lineage-negative cell; KSLc–, Kit^{+}/Sca-1^{+}/LN–; Sca-1, stem cell antigen-1; GM, granulocyte macrophages; SDF-1, stromal-cell-derived factor-1; ac-LDL, acetylated LDL; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.diabet.2016.07.034
1262-3636/

However, while the colony-forming capacity of EPCs and underlying mechanisms of EPC dysfunction are as yet not fully understood, their elucidation could aid the development of EPC-recovery-based therapies for microvascular and other ischaemic diseases in T2D patients, as the colony-forming potential of EPCs is important for angiogenic therapy.

EPCs comprise cells at a variety of differentiation levels, ranging from haemangioblasts to fully differentiated endothelial cells, and can be classified into various stages according to their level of differentiation [14]. Recently, EPC colony-forming assay (EPC-CFA), a novel method for assessing the colony-forming potential of EPCs at different differentiation levels, was established for human cord blood, peripheral blood and bone marrow (BM) [15,16]. Also, accumulating evidence from EPC-CFA has reported two types of EPC colony-forming units (CFUs) [15,16]: small EPC CFUs, which are 'primitive' EPCs; and large EPC CFUs, which are 'definitive' EPCs. More important, multiple reports have clearly shown the successful transplantation of large EPCs, but not of small EPCs, suggesting that large EPC CFUs have provasculogenic potential, whereas small EPC CFUs do not [16–18].

Thus, the present study aimed to examine the colony-forming capacity of EPCs and differentiation potential of bone marrow c-Kit(+)/Sca-I(+) lineage-negative mononuclear cells (BM-KSL) in T2D mice, *db/db* mice and KKA^{y} mice, using EPC-CFA. As BM-KSL have the plasticity to transdifferentiate into various cell types and given the close link established between the pathogenesis of T2D and inflammation, our study concomitantly investigated both the differentiation of BM-KSL into haematopoietic cells and development of vascular lineage cells in T2D mice.

2. Methods

2.1. Animals

Experiments were performed with male 9- to 11-week-old *db/+* and *db/db* mice, and male 10- to 11-week-old C57BL/6J and KKA^{y} mice (CLEA Japan, Inc., Tokyo, Japan), which were maintained under a 12-h light/dark cycle and in accordance with the regulations of Tokai University. Standard laboratory chow and water were available *ad libitum*. Mean body weight and blood glucose levels of the *db/+*, *db/db*, C57BL/6J and KKA^{y} mice were, respectively: *db/+*: 28.0 ± 1.0 g and 142 ± 39 mg/dL; *db/db*: 44.5 ± 2.6 g ($P < 0.01$ vs. *db/+* mice) and 356 ± 52 mg/dL ($P < 0.01$ vs. *db/+* mice); C57BL/6J: 24.4 ± 0.6 g and 155 ± 34 mg/dL; and KKA^{y}: 41.4 ± 3.3 g and 384 ± 68 mg/dL ($P < 0.01$ vs. C57BL/6J mice for both).

2.2. Isolation of peripheral blood mononuclear cells (PB-MNCs) and BM-KSL

Peripheral blood was obtained from the heart immediately before sacrifice and separated by Histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation, as previously described [19]. Briefly, low-density mononuclear cells were harvested and washed twice with Dulbecco's phosphate-buffered saline (PBS), supplemented with 2 mmol/L EDTA. Contaminated red blood cells were haemolyzed using an ammonium-chloride solution.

Bone marrow mononuclear cells (BM-MNCs) were obtained by flushing the femurs and tibias. Cells were exposed to a reaction mixture of biotinylated monoclonal antibodies (BD Biosciences, San Jose, CA, USA) against B220 (RA3-6B2), CD3 (145-2C11), CD11b (M1/70), TER-119 (Ly-76) and Gr-1 (RB6-8C5), used as lineage markers to deplete lineage-positive cells from BM-MNCs, with an autoMACS cell-separator system (Becton Dickinson, Franklin Lakes, NJ, USA). Lineage-negative bone marrow cells (BM-LN–) were incubated with saturating concentrations of directly labelled anti-c-Kit, anti-Sca-1 and anti-CD34 antibodies (all from BD Biosciences) for 30 min on ice. The c-Kit+/Sca-1+ lineage-negative (BM-KSL) or CD34(+) BM-KSL or CD34(–) BM-KSL were then isolated by live sterile cell-sorting (FACSVantage SE flow cytometry system; Becton Dickinson).

2.3. EPC differentiation assay in vitro

Modified EPC culture assay was performed as previously described [17]. In brief, BM-KSL (1×10^4 cells/well) were cultured in serum-free endothelial basal medium (EBM)-2 (Cambrex Bio Science Walkersville, Walkersville, MD, USA) with rat plasma vitronectin (Sigma-Aldrich) in a 0.5% gelatin solution and a 96-well plate (BD Biosciences). Four days after culture, cells were washed twice and incubated at 37 °C for 3 h in a medium containing 10 μg/mL 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine perchlorate-labelled acetylated low-density lipoprotein (DiI-ac-LDL; Biomedical Technologies Inc., Stoughton, MA, USA) and 20 μg/mL of fluorescein isothiocyanate (FITC)-conjugated BS-1 lectin (Sigma-Aldrich). After washing, cells were examined by fluorescence microscopy, with DiI-ac-LDL and BS-1 lectin double-positive cells counted in at least four separate, randomly selected, high-power fields.

2.4. EPC-CFA

EPC-CFA was performed as previously described [16]. Briefly, cells were cultured in a methylcellulose-containing medium (MethoCult^{TM} SF M3236, StemCell Technologies, Vancouver, BC, Canada) with 20 ng/mL stem cell-derived factor (Kirin-Sankyo Co. Ltd, Tokyo, Japan), 50 ng/mL vascular endothelial growth factor (R&D Systems, Inc., Minneapolis, MN, USA), 20 ng/mL interleukin-3 (Kirin-Sankyo), 50 ng/mL basic fibroblast growth factor (Wako, Osaka, Japan), 50 ng/mL epidermal growth factor receptor (Wako, Osaka, Japan), 50 ng/mL insulin-like growth factor-1 (Wako), 2 U/mL heparin (Ajinomoto Co., Inc., Tokyo, Japan) and 10% FBS in a 35-mm dish for 8 days. Cell densities for each sample were: PB-MNCs: 7×10^5 cells/dish; KSL: 500 cells/dish; and CD34(+) or CD34(–) BM-KSLs: 500 cells/dish. EPC CFUs were identified as large CFUs or small CFUs by visual inspection, using an inverted microscope under × 40 magnification. Large CFUs