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RESEARCH ARTICLE

Characterization of endothelial progenitor cells in the NOD mouse as a source for cell therapies

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Abstract

Background Endothelial progenitor cells (EPCs) in bone marrow (BM) and peripheral blood (PB) contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels. Previous studies indicate that diabetic patients have reduced EPC number and deregulated EPC function, although the regenerative properties of EPCs in diabetes are unknown. We wish to characterize and compare EPCs from pre-diabetic and diabetic non-obese diabetic (NOD) mice, a model of type 1 diabetes (T1D), in order to delineate the role of these cells in the pathogenesis of autoimmune diabetes.

Methods Whole BM was obtained by flushing femurs, tibias and illiac crests from pre-diabetic and diabetic NOD mice (5–30 weeks) in which the diabetic status was confirmed by measuring blood glucose levels ($\geq$11.5 mmol/L); PB was collected in heparin-coated tubes and lysed after incubation with antibodies directed against EPCs.

Results FACS analyses revealed a significant decrease in EPC number ($CD31^+$, c-Kit^+, Sca-1^+, Lin^-) in BM from diabetic compared to pre-diabetic mice ($P = 0.02$). Conversely, EPC number was significantly increased in PB from diabetic compared to pre-diabetic mice ($P = 0.01$).

Conclusions These data suggest that at the onset of diabetes, BM-derived EPCs are stimulated to enter the systemic circulation likely in response to signals from the pancreas. Further studies are required to elucidate whether EPCs home the damaged pancreas, thus representing a prospective source of autologous cells for β-cell regeneration therapy.

Keywords autoimmune diabetes; endothelial progenitor cells; bone marrow cells; NOD mouse

Introduction

The successful isolation of endothelial progenitor cells (EPCs) in 1997 by Asahara *et al*. [1] has led to many studies to date, and it is now well known that EPCs derived from bone marrow (BM) and peripheral blood (PB) contribute to tissue repair in various pathological conditions *via* the formation of new blood vessels, i.e. neovasulogenesis. EPCs are believed to originate from the BM and are often mobilized into the circulation in response to growth factors and cytokines released following a variety of stimuli which include vascular trauma [2], ischaemia, wounding or cancer [3]. EPCs home-in and incorporate into sites of damage and mediate repair by inducing neovascularization.

Recent studies on angiogenesis have revealed a significant impairment of angiogenesis in diabetic patients [4,5]. Additionally, these patients have a reduced number of PB EPCs [5,6]. Loomans *et al*. showed that this endothelial dysfunction does not occur due to increased apoptosis but perhaps is associated with alterations in BM mobilization and hyperglycaemic stress [5]. Parallel studies have been carried out in ischaemic [7] and induced diabetic animal models [4,8] but has yet to be carried out on the most valuable spontaneous model oftype 1 diabetes (T1D) such as the non-obese diabetic (NOD) mouse [9].

Accordingly, the aim of this study was to characterize and compare BM and PB EPCs from pre-diabetic and diabetic NOD mice, in order to delineate their fate in the natural history of autoimmune diabetes.

Materials and methods

Mice

Two strains of mice were used in this study. The first strain, NOD mice came from the NOD/Ba colony established in 1987 at St. Bartholomew's Medical College, London, UK, originally derived from Dr E. Leiter's laboratory (Bar Harbor, ME, USA). There is a stable cumulative incidence of diabetes of approximately 60% in female and 15% in male mice at 30 weeks of age [10]. The colony is housed in a purpose-built area and maintained strictly according to international [11] and United Kingdom [12] guidelines for animal care. Diabetic status of the mice was initially diagnosed by monitoring urinary glucose level (Diabur Test 5000, Boehringer Mannheim, Germany) and then confirmed by measuring blood glucose levels ($\geq$11.5 mmol/L) using Accu-chek AVIVA Blood Monitor (Roche Diagnostics GmbH, Mannheim, Germany). NOD mice were investigated at different ages ranging from 5 to 30 weeks including diabetic mice. Diabetic NOD mice aged 18 weeks onwards were first confirmed to be diabetic in order to evaluate the effect of hyperglycaemia on the fate of BM and PB EPCs as compared to non-diabetic age-matched mice. Balb/c mice were obtained from Charles River Laboratories (Charles River Laboratories, Wilmington, ME, USA). Only female NOD mice were enrolled in this study. All mice were sacrificed by cervical dislocation.

Blood collection

A single blood aliquot was collected by cardiac puncture from the ventricle under terminal halothane anaesthesia using appropriate size needles and collected into heparin-coated tubes (Sarstedt, Numbrecht, Germany). Fresh blood collected in heparin-coated tubes were mixed thoroughly and spun down briefly. Blood was stored on ice and used within 2 h of collection.

Isolation of whole BM cells

After cervical dislocation, the femurs, tibias and iliac crests were dissected out using a pair of sterile sharp scissors and pincers. Ligaments and excess tissue were removed using a disposable scalpel (Swann-Morton, Sheffield, UK.), cleaned and collected in Bijou tubes containing sterile X1 PBS. BM cells were collected by flushing the bones with Iscove's MDM with 2% Fetal Bovine Serum (FBS) (Stem Cell Technologies, Vancouver, Canada) using a 23G needle (Neolus Terumo Corporation, Madrid, Spain). After each flushing of a part of the bone, the cells were collected in a 5 mL Falcon tube and were mixed using a 21G needle (BD Microlance 3, Becton-Dickinson, Bedford, USA) several times to prevent coagulation. The cells were then filtered with a 70 μm cell strainer (Becton-Dickinson, Bedford, USA) into another 5 mL Falcon tube and centrifuged (Hettich Zenrifugen Microcentrifuge, Jensons-Pls, Bedfordshire, UK) at 1500 rpm for 6 min at 4 °C. The resulting pellet was re-suspended in 1 mL of cold 1% FBS PBS and kept on ice. The cells were counted using a haemocytometer and diluted with X1 PBS to obtain 1×10^6 cells to be next used for FACS analysis.

FACS analysis

FACS analysis was carried out to quantify the number of EPCs and circulating EPCs from BM and PB, respectively.

Whole BM cells

Hematopoietic stem cells, natural killer cells, erythrocytes and macrophages were defined by negative staining (lin^-) with lineage panel antibodies (CD23, CD45, CD11b, and Ter119; BD Pharmingen, San Diego, CA). EPCs were defined by positive staining for c-Kit (BD Pharmingen), stem cell antigen 1 (Sca-1; BD Pharmingen) and CD31 (BD Pharmingen). Appropriate fluorochrome-conjugated isotype controls were used for each staining procedure.

Whole blood

A volume of 100 μL PB was incubated for 15 min in the dark with Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against mouse CD45 (BD Pharmingen) in combination with Allophycocyanin (APC)-conjugated antibody against mouse c-Kit (BD Pharmingen), PE-Cy7-conjugated antibody against mouse Sca-1 (BD Pharmingen) and PE-conjugated antibody against CD31 (BD Pharmingen). Isotype-identical antibodies served as controls (BD Pharmingen). After incubation, erythrocytes in PB were lysed using FACS lysing solution (Becton-Dickinson, Bedford, USA) and washed with PBS before analysis. Each analysis included approximately 10 000 events.

Analysis-gating strategies

Whole BM and PB cells (approximately 1×10^6 cells) were analysed with the fluorescence-activated cell sorter

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(FACS) Aria and BD LSR II, respectively (Becton-Dickinson, San Jose, CA, USA). Single colour and negative selection antibodies with isotype controls were analysed first. This was followed by the analysis of cells labelled with lineage-positive (c-Kit$^+$ CD31$^+$ Sca1$^+$) and lineage-negative antibodies (lin$^-$). Compensation and gating strategies were performed by firstly gating c-Kit$^+$ lin$^-$ cells. These cells were then gated to include only CD31$^+$ Sca1$^+$ cells. Only single cells of c-Kit$^+$ CD31$^+$ Sca1$^+$ lin$^-$ were obtained and located in the mononuclear region of a forward scatter (FSC) *versus* side scatter (SSC) graph.

Statistics

Results are expressed as means ±SEM. Statistical significance of differences between EPC numbers (%) was assessed using Student's unpaired *t*-tests.

Results

We measured the number of EPCs from BM and PB using FACS analysis. FACS analysis was first carried on BM and PB of wild type (Balb/c) and non-diabetic NOD mice. To obtain a single-cell population of EPCs, c-Kit$^+$ Lin$^-$ cells were first gated and subsequently gated for CD31$^+$ and Sca-1$^+$ cells. FACS analyses revealed that there was no significant difference in EPC number (%) ($n = 5$) (CD31$^+$, c-Kit$^+$, Sca-1$^+$, Lin$^-$ cells) in BM and blood from non-diabetic NOD mice compared to WT (Balb/c) mice as shown in Figure 1. In the following experiments, BM and blood from non-diabetic NOD mice served as controls, and the same gating strategies were used.

Using the same gating strategies, FACS analyses were carried out to compare the number of BM and PB EPCs in the diabetic and non-diabetic NOD mice. Figures 2 and 3 show the representative FACS gating strategies to obtain BM and blood EPCs (CD31$^+$, c-Kit$^+$, Sca-1$^+$, Lin$^-$), 2 respectively. The results demonstrated a significant decrease in EPC number (CD31$^+$, c-Kit$^+$, Sca-1$^+$, Lin$^-$) in BM from diabetic compared to non-diabetic NOD mice

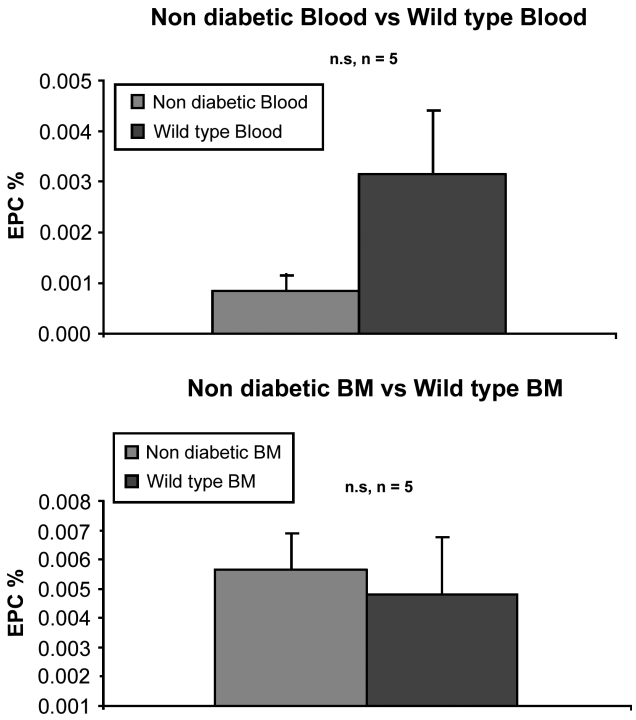

Figure 1. EPC numbers (%) comparison (BM and blood) between non-diabetic NOD and WT (Balb/c) mice. EPCs (c-Kit$^+$ CD31$^+$ Sca1$^+$ lin$^-$) were obtained using flow cytometry. All experimental data are expressed as means ± SEM of respective number of experiments, using two types of mice ($n = 5$) (non-diabetic NOD mice *versus* Balb/c mice). This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

($P = 0.02$) (Figure 4). Conversely, EPC number (%) was significantly increased in PB from diabetic [compared to non-diabetic NOD mice ($P = 0.01$)].

Discussion

In the present study, we have characterized and isolated EPCs from the BM and PB of Balb/c and NOD mice. To characterize EPCs from the BM and blood, c-kit, CD31 and Sca-1 markers were used. c-Kit is a primordial stem

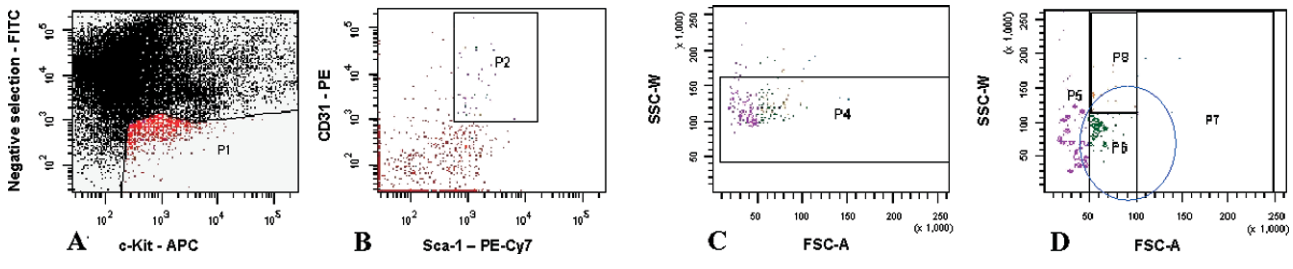

Figure 2. Characterization of EPCs (CD31$^+$, c-Kit$^+$, Sca-1$^+$, Lin$^-$) from whole BM cells (1 x 10^6 cells). Cells were analysed on a BD FACSAria. Single colour and negative selection antibodies with isotype controls were also analysed. (A) Compensation and gating strategies were performed by firstly gating c-Kit$^+$ lin$^-$ (APC +ve and FITC −ve cells), (B) CD31$^+$ Sca1$^+$ cells (PE and PE-Cy7 +ve cells) were then gated from c-Kit$^+$ lin$^-$ cells, (C) A band of uniform expressing APC +ve cells were then gated against FSC and doublet discrimination achieved by use of side scatter width (SSC-W) parameter against FSC, (D) These events were then back-gated against forward side scatter (FSC) *versus* SSC to obtain single cells of c-Kit$^+$ CD31$^+$ Sca1$^+$ lin$^-$ which are located in the mononuclear region of the FSC *versus* SSC graph

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marker and is important in EPC mobilization [13]. Sca-1 is a well known progenitor stem cell marker and is important in the regulation of c-Kit. The third marker used in this study was CD31/PECAM-1 suggested to be a marker for early angiogenesis [14] as well as the earliest marker of endothelial cell differentiation [15]. To isolate a purified population of EPC cells, lineage-negative antibodies (CD23, CD11b, TER119 and CD45) were used to eliminate platelets, natural killer cells, macrophages and leukocytes. In our study, fresh cells were isolated from BM and PB. In contrast to other studies that used enriched mononuclear fractions [5,16], we have demonstrated that EPC or EPC-like cells can be isolated from fresh whole BM and PB although EPC populations are small. When cells were pooled and analysed using flow cytometry, a six-fold difference between diabetic and non-diabetic BM EPCs was noted (data not shown) which is in line with our FACS analysis data in which we demonstrated a reduction of BM EPCs in the diabetic mice and an increase of circulating EPCs in the blood (Figures 3 and 4).

Based on these preliminary data, BM-derived EPCs may have been stimulated to enter the systemic circulation in response to signals from the pancreas, although this hypothesis requires more evidence. Further studies thus need to be carried out to investigate whether EPCs can home to the damaged pancreas and contribute to β-cell regeneration. Mathews *et al*., have demonstrated that BM-derived EPCs could be stimulated to enter the systemic circulation in response to signals from the pancreas [17]. This latter observation is supported by several studies of BM-derived stem cell engraftment and contribution to neovasculogenesis in adult tissues [18–23], which is enhanced by increasing regenerative demand within a diseased or damaged tissue. The exact signalling pathways and molecular mechanism for this EPC recruitment are as yet unknown.

Human studies have demonstrated a reduction of EPCs in the PB of diabetics [5]. However we observed that there is an increase of circulating EPCs in diabetic mice compared to non-diabetic NOD mice. This could be attributed to the extent of pancreatic damage of this disease model in which the mice used in the study have been diabetic for 2 weeks. Besides their ability to migrate and differentiate into endothelial cells when recruited, there is existing evidence suggesting that EPCs have paracrine effects in which EPCs release cytokines or growth factors to contribute to tissue regeneration at a damaged site. There is a possibility that the EPCs were recruited to the injury site during the pre-diabetic stages and successfully engrafted with the aid of the cytokines released to aid regeneration of the pancreas. However, as shown in humans, the EPCs in diabetic mice may be dysfunctional, and thus, they would be unable to completely vascularize or regenerate the damaged site.

The progression of autoimmune T1D in mice and humans are characterized by infiltration of inflammatory cells and increasing blood glucose levels. Cytokines

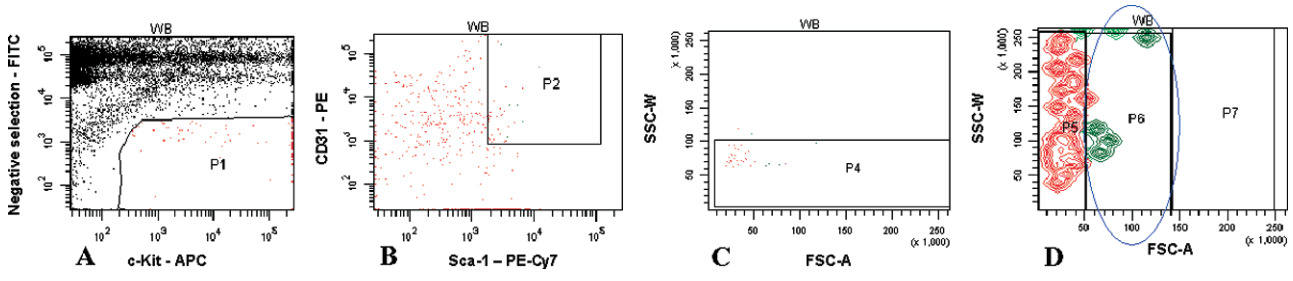

Figure 3. Characterization of EPCs ($CD31^+$, c-Kit^+, Sca-1^+, Lin^-) from PB cells (<0.5 x 10^6 cells). Heparinized and lysed PB cells were analysed on a BD LSRII. Single colour and negative selection antibodies with isotype controls were also analysed. (A) Compensation and gating strategies were performed by firstly gating c-Kit^+ lin^- (APC +ve and FITC −ve cells), (B) $CD31^+$ $Sca1^+$ cells (PE and PE-Cy7 +ve cells) were then gated from c-Kit^+ lin^- cells, (C) A band of uniformly expressing APC +ve cells were then gated against FSC and doublet discrimination achieved by use of SSC-W parameter against FSC, (D) These events were then back-gated against FSC *versus* SSC to obtain single cells of c-Kit^+ $CD31^+$ $Sca1^+$ lin^- which are located in the mononuclear region of the FSC *versus* SSC graph

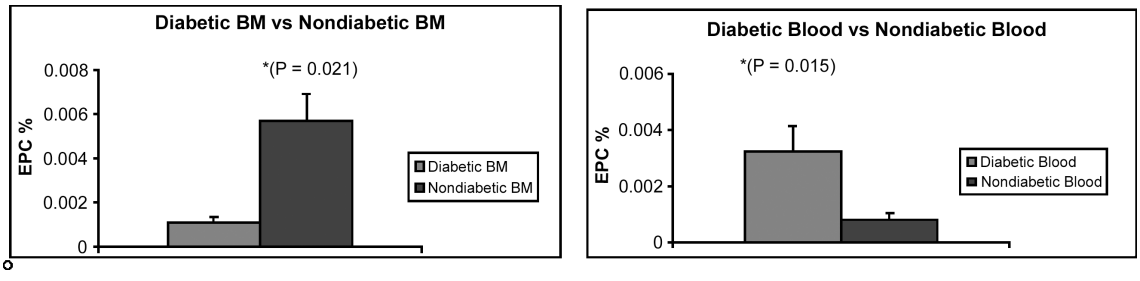

Figure 4. EPC numbers (%) are plotted based on the average EPC % obtained from FACS analyses from five non-diabetic and five diabetic NOD mice. All experimental data are expressed as means ± SEM of respective number of experiments, using two types of mice (diabetic *versus* non-diabetic mice). This figure is available in colour online at www.interscience.wiley.com/journal/dmrr

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such as IL-1β can be released during the inflammation process in the islets. Amano *et al*., have shown that IL-1β can increase vascular cell adhesion molecule (VCAM)-1 expression on endothelial cells [24] and thus, may play a role in mobilization and recruitment and homing-in of EPCs in response to the signals from the pancreas. Further studies are thus warranted to investigate the relationship between insulitis and EPC recruitment.

Various methods have been used to characterize and isolate EPCs from blood or BM, which includes their ability to proliferate, adhere, form tubules and migrate in cell culture. However, debate still ensues as to the true phenotype of adult EPCs, which is often clouded by the overlap of the phenotype of EPC cells with haematopoietic stem cells (HSCs) and endothelial cells, and thus, a universally accepted EPC phenotype remains to be achieved (reviewed in [25]). These approaches are further hampered by the fact that only a small number of cells can be isolated from blood or BM [26].

In conclusion, our data showed that in the best model of autoimmune diabetes there is a mobilization of EPCs from BM into the circulation at the time of diabetes development. The role of these cells in β-cell regeneration therapy should be investigated in the future.

Acknowledgements

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Conflict of interest

The authors have no conflicts of interest.

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