Diabetes alters subsets of endothelial progenitor cells that reside in blood, bone marrow, and spleen

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Saito H, Yamamoto Y, Yamamoto H. Diabetes alters subsets of endothelial progenitor cells that reside in blood, bone marrow, and spleen. Am J Physiol Cell Physiol 302: C892–C901, 2012. First published December 7, 2011; doi:10.1152/ajpcell.00380.2011.—Circulating endothelial progenitor cells (EPCs) derived from the bone marrow (BM) participate in maintaining endothelial integrity and vascular homeostasis. Reduced EPC number and function result in vascular complications in diabetes. EPCs are a population of cells existing in various differentiation stages, and their cell surface marker profiles change during the process of mobilization and maturation. Hence, a generally accepted marker combination and a standardized protocol for the quantification of EPCs remain to be established. To determine the EPC subsets that are affected by diabetes, we comprehensively analyzed 32 surface marker combinations of mouse peripheral blood (PB), BM, and spleen cells by multicolor flow cytometry. Ten subsets equivalent to previously reported mouse EPCs significantly declined in number in the PB of streptozotocin-induced diabetic mice, and this reduction was reversed by insulin treatment. The PI-Lin−c-Kit+Sca-1+Flk-1−CD34−CD31+ EPC cluster, which can differentiate into mature endothelial cells in vitro, was the highest population in the PB, BM, and spleen and occurred 61 times more in the spleen than in the PB. The cell number significantly decreased in the BM as well as in the PB but paradoxically increased in the spleen under diabetic conditions. Insulin treatment reversed the decrease of EPC subsets in the BM and PB and reversed their increase in spleen. A similar tendency was observed in some of the major cell populations in db/db mice. To the best of our knowledge, we are the first to report spatial population changes in mouse EPCs by diabetes in the blood and in the BM across the spleen. Diminished circulating EPC supply by diabetes may be ascribed to impaired EPC production in the BM and to decreased EPC mobilization from the spleen, which may contribute to vascular dysfunction in diabetic conditions.

flow cytometry; endothelial progenitor cell subsets; diabetic vascular complications

IN 1997, ASAHARA ET AL. (3) reported the isolation of putative endothelial progenitor cells (EPCs), which could differentiate into endothelial cells (ECs) in vitro and in vivo, from adult human peripheral blood (PB). EPCs are derived from the bone marrow (BM) and play an important role in the regulation of vascular homeostasis and formation of new blood vessels (5). Clinically, the number and function of EPCs have been suggested as surrogate markers of endothelial function and cardiovascular disease (32). EPC therapy has also been studied as a new strategy in regenerative medicine (2). Various methodologies have been reported for EPC identification and isolation, as follows: 1) culture methods using fibronectin-coated plates with endothelial growth factors, 2) flow cytometry with antibodies against cell surface markers, and 3) in vitro colony formation. However, the definition of EPCs with angiogenic potential by cell surface markers is challenging because their surface marker profiles change during the process of mobilization and maturation. EPCs also share many stem and progenitor cell markers with hematopoietic stem cells (HSC) in the BM. EPCs may therefore encompass a group of cells existing in a variety of stages ranging from hemangioblasts to fully differentiated ECs. A generally accepted marker combination and a standardized protocol with regard to the reagents and gating strategies are not yet established for the quantification of EPCs. Although many researchers use markers of lineage (Lin), c-Kit, Sca-1, Flk-1, CD34, and CD31 for the identification of mouse EPCs (4, 6, 9, 10, 11, 12, 15, 17, 20, 21, 23, 24, 26, 31), Yang et al. (36) recently reported that CD34 solely would be a better and more suitable marker for mouse functional EPCs, with more homing and vasculogenic properties as compared with other commonly used markers—Lin−c-Kit+Sca-1+, Lin−c-Kit+, and Lin−Sca-1+ cells (36). The BM is a producing center and an important reservoir of EPCs, and it gives rise to various lineage-committed cells (22). The spleen also may function as a reservoir of EPCs, for example, as a storage site for inflammatory monocytes (29). Despite their potentially pivotal function and activity, the cellular events of EPCs in the BM and spleen under disease conditions are surprisingly unknown.

Vascular endothelial function is impaired in patients with diabetes (7). An epidemiological study showed that patients with type 2 diabetes have a two- to threefold increased risk of cardiovascular events compared to subjects without type 2 diabetes (14). Diabetes is directly implicated in various cardiovascular diseases, including stroke, ischemic heart disease, and peripheral vascular diseases. Reduced availability and downregulation of EPCs are among the several important mechanisms that are responsible for the occurrence of endothelial dysfunction and vascular disease in diabetes (37). Emerging evidence suggests that both type 1 diabetes and type 2 diabetes are associated with reduced numbers and impaired function of EPCs (13, 19). Hyperglycemia-induced oxidative stress has been suggested as a potential mechanism for reduced EPC count and EPC impairment (35, 37). However, it is still unknown what types of EPCs or whether all types of EPCs, as characterized by cell surface markers, are affected in the PB by diabetes and in the BM and spleen as well.

In this study, we have classified mouse mononuclear cells into 32 repertoires by using a combination of typical cell surface markers, including Lin, c-Kit, Sca-1, Flk-1, CD34, and CD31, and we then comprehensively analyzed the EPC subclasses by multicolor flow cytometry. To identify the EPC...
subclasses that can be affected by diabetic conditions, the cell number of each repertoire was counted in PB, BM, and spleen and then compared among streptozotocin (STZ)-induced diabetic mice with or without insulin treatment, db/db mice, and age-matched nondiabetic controls.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (Charles River) of 8 wk of age were purchased. For induction of diabetes, the mice were fasted for 4 h and then injected intraperitoneally with STZ (50 mg/kg body wt; Sigma-Aldrich, St. Louis, MO) in sodium-citrate buffer (pH 4.5). This procedure was conducted for five consecutive days (18, 33). After confirmation of hyperglycemia and a diabetic state, the STZ-induced diabetic mice were divided into two groups: those that received insulin injection (insulin detemir) subcutaneously twice a day for 2 or 5 wk and those that were not administered insulin. Male C57BLKS-LEprdb/LEprdb (db/db) mice were used as type 2 diabetes models. Animals were treated in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University.

Preparation of PB, BM, and spleen cells. Whole blood was drawn from the right ventricle into collection tubes containing EDTA at a final concentration of 5 mM. Red blood cells (RBCs) in the collected blood were lysed with 1× RBC Lysis Buffer (eBioscience), filtered through a 40-μm mesh (BD Biosciences), and centrifuged. The remaining cells in the pellet were resuspended in fluorescence-activated cell sorting (FACS) staining buffer (PBS containing 1 mM EDTA and 2% FBS) (34). BM cells were isolated from the femur and tibia of each hindleg. Whole spleen was mashed through a stainless steel strainer. BM and spleen cells were collected into tubes containing ice-cold FACS staining buffer, filtered through a 40-μm mesh (BD Biosciences), pelleted by centrifugation, and resuspended again in ice-cold FACS staining buffer. For subsequent cultivation, PB, BM, and spleen cells were purified by the Ficoll-Paque PLUS method (GE Healthcare) (25). All steps were performed under aseptic conditions.

Flow cytometry and cell sorting. Qualification and quantification of EPCs in PB, BM, and spleen were conducted by multicolor flow cytometry. Isolated cells were treated with FcBlock (BD Biosciences) and incubated for 15 min at 4°C (34). Cells were then stained with the following antibodies (for 15 min at 4°C in the dark): Lineage (Lin)-APC (BD Biosciences), CD31-PE-Cy7 (BioLegend), CD34-biotin (eBioscience), Sca-1-PE (BioLegend), Flk-1, CD34, and CD31: CD31/platelet-EC adhesion molecule-1 (PECAM-1) and Flk-1/CD309 (vascular endothelial growth factor receptor 2) for mature ECs; CD34 and c-Kit/CD117 for hematopoietic stem/progenitor cells; and Sca-1 (Ly-6A/E) for stem cells (4, 6, 9, 10, 11, 12, 15, 17, 20, 21, 23, 24, 26, 31). Figure 2A illustrates the strategies for analyzing protein (DiL-Ac-LDL), and chemical binding of isoleucin B4, were assessed by confocal microscopy (Carl Zeiss). Briefly, adherent cells were incubated with 10 μg/ml DiL-Ac-LDL for 4 h at 37°C under 5% CO2. After washing with culture medium, the cells were further incubated with 10 μg/ml Alexa Fluor 488-labeled isoleucin B4 (Invitrogen) for 1 h at 37°C under 5% CO2. The cells were washed with PBS and then fixed with 4% paraformaldehyde at room temperature. Nuclei were counterstained with DAPI. ECs were defined as cells doubly positive for DiL-Ac-LDL and isoleucin B4 (27). Human umbilical vein ECs (HUVEC) were used as a positive control.

Tube formation assay. HUVEC and sorted cells were used for tube formation assays as described previously (36). Sorted cells from the PB, BM, and spleen were stained with PKH2 green fluorescent cell linker (Sigma-Aldrich). After washing with conditioned media, PKH2-labeled cells were mixed with HUVEC and then seeded onto Matrigel-coated eight-well Lab-Tek chamber slides in 1% FBS-EBM-2 medium (Lonza). After 24 h in culture, PKH2-labeled cells incorporated into tubelike structures formed with HUVEC were observed under fluorescent microscopy.

Statistics: All values are expressed as means ± SE. Comparisons among groups were analyzed by Student’s t-test or ANOVA combined with a multiple-comparison test (Scheffe’s type). These analyses were carried out with the use of StatView V software (SAS institute).

RESULTS

Diabetic mouse models. To observe diabetes-induced changes in EPCs in PB, BM, and spleen, we used multiple low-dose STZ-injected (STZ-diabetic) mice and genetically obese db/db mice as type 1 and type 2 diabetes models, respectively. To rule out systemic and direct cytotoxic effects of STZ in the evaluation of diabetes-mediated insults, mice made euglycemic by twice-a-day subcutaneous insulin injections (STZ-nondiabetic) were used as another control model. We also performed analyses at different time points. Utmost care was taken in the interpretation of results from the STZ-diabetic models. In STZ-diabetic mice, fasting blood glucose (FBG) levels significantly increased to 375 ± 24 and 379 ± 25 mg/dl compared with nondiabetic controls (67 ± 4 and 122 ± 11 mg/dl at 2 and 5 wk, respectively, after the induction of diabetes) (Fig. 1B). Insulin treatment significantly decreased FBG levels compared with STZ-diabetic mice at both 2 and 5 wk after diabetes induction (Fig. 1). The db/db mice also showed hyperglycemia, with 203 ± 20 mg/dl FBG at 14 wk of age (Fig. 1B). Fasting body weight in STZ-diabetic mice was significantly less than that in nondiabetic controls, and insulin treatment restored body weight loss (Fig. 1A). Lower spleen weight was also observed in the STZ-diabetic group at 5 wk after diabetes induction, which recovered with insulin treatment (Fig. 1D). However, the spleen weight-to-body weight ratio did not change in either STZ-diabetic mice or db/db mice which was represented by obesity and heavier spleen weight (Fig. 1C).

Comprehensive analysis of mouse EPCs by flow cytometry. Multicolor flow cytometric analysis was conducted to classify PT live cells with 32 combination repertoires of the cell surface markers for mouse EPCs, including Lin, c-Kit, Sca-1, Flk-1, CD34, and CD31: CD31/platelet-EC adhesion molecule-1 (PECAM-1) and Flk-1/CD309 (vascular endothelial growth factor receptor 2) for mature ECs; CD34 and c-Kit/CD117 for hematopoietic stem/progenitor cells; and Sca-1 (Ly-6A/E) for stem cells (4, 6, 9, 10, 11, 12, 15, 17, 20, 21, 23, 24, 26, 31). Figure 2A illustrates the strategies for analyzing
Fig. 1. Metabolic phenotype of diabetic animal models. A: fasting body weight. B: fasting blood glucose (FBG). C: fasting spleen weight/fasting body weight (mg/g). D: fasting spleen weight. C, age- and sex-matched control mice (n = 6); D, STZ-induced diabetic mice at 2 or 5 wk (w) after diabetes induction (n = 6); D + I, STZ-induced diabetic mice that received insulin 5 days after diabetes induction (11 and 14 wk of age, respectively) (n = 6); db, db/db mice at 14 wk of age. ns, not significant. Values represent means ± SE. *P < 0.05; **P < 0.01.

and gating PB cells. Mononuclear cell gating was done using scatterplots of forward-scatter versus side-scatter measurements. Cell aggregates, PI⁺ dead cells, and Lin⁻ cells were then excluded; the classification into 32 groups was based on positive or negative staining for CD31/CD34, Flk-1/Sca-1, and c-Kit (Fig. 2A). The same gating procedures were done for BM and spleen cells, as shown in Fig. 2, B and C, respectively. Figure 3, A, C, and D, represents relative cell number (per 10⁶ live cells), and Fig. 3, B and E, shows the total cell number of the 32 classified repertories of PB, BM, and spleen cells from nondiabetic, STZ-diabetic, and STZ-nondiabetic mice at 5 wk after diabetes induction. The following 10 subclasses were categorized as mouse EPCs in the PB according to previous reports (5–19):

Lin⁻ c-Kit−Sca1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, and Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺, and Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ and Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ cells were the second and third largest populations, respectively (Fig. 3, A and B). STZ-diabetes decreased the cell ratio and total cell number in almost all 10 subsets, and the reduction was reversed by insulin treatment (Fig. 3, A and B). Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ cells were also the most abundant in the BM and the spleen, constituting 2.5% of live BM cells and 1.9% of spleen-derived cells. The total number of spleen Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ cells was 123,099 under normal healthy conditions (Fig. 3, C and D). This indicates that there were 61 times more EPCs in the spleen than in the PB, suggesting a functional role of spleen as an EPC reservoir. The relative number of Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ and Lin⁻ c-Kit−Sca-1⁺Flk-1⁺CD34⁺CD31⁺ cells increased by 10.220.33.2 on October 29, 2017 http://ajpcell.physiology.org/ Downloaded from
1−CD34+CD31+—were significantly increased by STZ-diabetes, an effect reversed by insulin treatment (Fig. 3, D and E). Total cell number per wet spleen weight ratio also showed similar results (Fig. 3F). Lin−c-Kit+Sca-1+CD34+ and Lin−c-Kit+Sca-1+CD31+CD34+ cells are known to be HSC in mice; Lin−c-Kit+Sca-1+Flk-1−CD34+CD31+ and Lin−c-Kit+Sca-1+Flk-1−CD34−CD31+ cells fit into this category and were also affected by STZ-diabetes and rescued by insulin in the PB and BM (Fig. 3,
Fig. 3. Cell number of endothelial progenitor cells (EPCs). A: relative cell number (per 10⁶) in peripheral blood (PB). B: total cell number in PB. C: relative cell number (per 10⁶) in bone marrow (BM). D: relative cell number (per 10⁶) in spleen. E: total cell number in spleen. F: total cell number in the spleen per spleen weight. Data for PI"Lin" CD31 "CD34" Flk-1 "Sca-1" "c-Kit" are not shown. White columns, nondiabetic controls; black columns, STZ-induced diabetes; gray columns, STZ-induced diabetes with daily insulin treatment 5 days after diabetes induction. Data shown are for 5 wk after STZ injection. Values represent means ± SE. *P < 0.05; **P < 0.01 between nondiabetic controls and STZ-induced diabetes or between STZ-induced diabetes and STZ-induced diabetes with insulin treatment.
In the spleen, Lin- c-Kit+ Sca-1+ Flk-1+ CD34+ CD31+ cells were not affected by STZ-diabetes (Figs. 3–5).

To determine the effects common to both type 1 and type 2 diabetes in the EPC populations, we analyzed PB, BM, and spleen cells from db/db mice, a type 2 diabetes model, in addition to STZ-diabetic mice. Age- and sex-matched control mice were also used. As a result, we were able to categorize four patterns of spatial and dynamic changes in mouse EPC subclasses (Figs. 4 and 5), as follows: A type, PB ↓↓ BM ↓↓ spleen ↑↑; A’ type, PB ↓↓ BM ↓↓ spleen ↑↓; A” type, PB ↓↓ BM ↓↓ spleen ↑↑; and B type, PB → BM ↓↓ spleen ↑↑ (former and latter arrows indicate STZ-diabetic and db/db mice, respectively). The A type includes Lin- c-Kit- Sca-1+ Flk-1+ CD34+ CD31- cells showing the same pattern in type 1 and type 2 diabetes. A’ type represents almost a similar pattern to A type but without the increase in spleen cell number in db/db mice; this was exemplified by Lin- c-Kit+ Sca-1+ Flk-1+ CD34- CD31+ cells. A” type is also categorized as a substrain of A type with increased BM cell number in db/db mice, including Lin- c-Kit- Sca-1+ Flk-1+ CD34+ CD31+ cells. A type is also categorized as a substrain of A type with increased BM cell number in db/db mice, including Lin- c-Kit+ Sca-1+ Flk-1+ CD34+ CD31+ cells. A” type is also categorized as a substrain of A type with increased BM cell number in db/db mice, including Lin- c-Kit- Sca-1+ Flk-1+ CD34- CD31+ cells. B type includes Lin- c-Kit- Sca-1+ Flk-1+ CD34- CD31- and Lin- c-Kit+ Sca-1+ Flk-1+ CD34+ CD31+ cells; both of these subclasses showed a decrease in STZ-diabetes and an increase in the BM and spleen cells in db/db mice. There were no EPC subclasses in the db/db spleen, which exhibited a decrease in total cell number compared with the control (Fig. 5). BM cells categorized as A’ and B types seemed to be compensatorily and responsively increased in number under obesity and leptin receptor signaling-deficient conditions in db/db mice (Fig. 4). The HSC cluster of Lin- c-Kit+ Sca-1+ Flk-1+ CD34+ CD31+ cells also showed similar responses in the BM (Fig. 4). Figure 6 shows the cumulative EPC number of the 10 subsets. STZ-diabetes significantly decreased and increased the total EPC number in the PB and spleen, respectively (Fig. 6). A substantial accumulation of splenic EPCs was also observed in db/db mice (Fig. 6).

Differentiation of sorted cells into mature ECs in culture and their incorporation into tubulike structures. We next examined by confocal microscopy whether sorted EPCs could differentiate into mature ECs in vitro. Lin- c-Kit+ Sca-1+ Flk-1+ CD34+ CD31+ cells were sorted from the PB, BM, and spleen of nondiabetic wild-type C57BL/6J mice and then cultured for 2 wk. All the cells from the PB, BM, and spleen were found to be double positive for Dil-Ac-LDL and isolectin B4, as were HUVEC (Fig. 7), indicating their ability to mature into ECs in vitro. The cells’ ability to be incorporated into tubulike structures was also assessed. HUVEC were employed to form tubulike structures. Sorted and PKH2-labeled Lin- c-Kit- Sca-1+ Flk-1+ CD34+ CD31+ cells were analyzed by confocal microscopy whether sorted EPCs could differentiate into mature ECs in vitro. Lin- c-Kit- Sca-1+ Flk-1+ CD34+ CD31+ cells were sorted from the PB, BM, and spleen of nondiabetic wild-type C57BL/6J mice and then cultured for 2 wk. All the cells from the PB, BM, and spleen were found to be double positive for Dil-Ac-LDL and isolectin B4, as were HUVEC (Fig. 7), indicating their ability to mature into ECs in vitro. The cells’ ability to be incorporated into tubulike structures was also assessed. HUVEC were employed to form tubulike structures. Sorted and PKH2-labeled Lin- c-Kit- Sca-1+ Flk-1+ CD34+ CD31+ cells were analyzed by confocal microscopy.
cells from the PB, BM, and spleen were found to be incorporated into tubelike structures (Fig. 8).

**DISCUSSION**

In this study, we comprehensively classified 32 repertoires of cell surface markers for mouse EPCs and analyzed their populations in the PB, BM, and spleen by multicolor flow cytometry. We focused on 10 subclasses, which fit into previously defined categories as mouse EPCs, and examined their quantitative changes in diabetes. The results obtained clearly demonstrated that all 10 subsets of EPCs decreased in number under STZ-diabetes in both the PB and BM and that this reduction was reversed by insulin treatment (Figs. 3–5). The cumulative number of the 10 subsets of EPCs in the PB also significantly decreased (Fig. 6). We examined the spatial changes of the EPC population in db/db mice, a type 2 diabetes model, in addition to

![Graphs showing data](image)

Fig. 5. Total cell number of EPCs. A–J: data corresponding to values in Fig. 4. Values represent means ± SE. *P < 0.05; **P < 0.01 between nondiabetic controls and STZ-induced diabetes or between nondiabetic controls and db/db mice. †P < 0.05; ‡P < 0.01 between STZ-induced diabetes and STZ-induced diabetes with insulin treatment.

![Graphs showing data](image)

Fig. 6. Total cell number of all EPC subsets in PB and spleen. Values represent means ± SE. *P < 0.05; **P < 0.01.
STZ-diabetic mice. The A type (Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{-}\)CD31\(^{-}\) cells) and A\(^{\prime}\) type (Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{-}\)CD31\(^{+}\), Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{+}\)CD31\(^{+}\), and Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{+}\)CD31\(^{+}\) cells) subsets showed a similar decrease pattern in the PB and BM in \(db/db\) mice (Figs. 4 and 5). The Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{-}\)CD31\(^{-}\) cluster was the most abundant population among the 10 subclasses in the PB, BM, and spleen (Fig. 3). The total number of Lin\(^{-}\)c-Kit\(^{-}\)Sca-1\(^{+}\)Flk-1\(^{-}\)CD34\(^{-}\)CD31\(^{-}\) cells was found to be about 61-fold higher in the spleen compared with that in the PB under healthy conditions (Fig. 3, B and E). In STZ-diabetes, this value significantly decreased by 15% in the PB at 5 wk after diabetes induction but paradoxically increased by 603% in the spleen (Fig. 4A), with the spleen versus PB EPC ratio reaching a value as high as about 2,400-fold. Unfortunately, we could not calculate the total cell number of EPCs in the BM because the collected BM cells represented only tibial and femoral cells and not whole marrow cells. A similar pattern of the change seen in the most
abundant population was observed in Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+, and Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+ cells (Figs. 4 and 5). However, the BM and splenic EPC patterns in db/db mice were not perfectly concordant with those in STZ-diabetic mice (Figs. 4 and 5). Especially, BM cells categorized as A~*~ and B type might be compensatorily increased in response to obesity or leptin receptor signaling-deficient conditions. Leptin receptor signaling is reported to be involved in hematopoiesis and EPC homing and migration but not in EPC proliferation (1, 28). We cannot fully explain the phenomena seen in db/db mice at this moment. The most abundant cluster other than the 10 subclasses, PI^-Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+ cells, showed similar population changes under diabetic conditions: a decrease in STZ-diabetes and the recovery by insulin treatment. Kim et al. (16) recently reported that single marker-positive (i.e., CD31-positive) cells in the PB and BM represent highly angiogenic and vasculogenic potential. However, we excluded the PI^-Lin^-c-KIT^-Sca-1^-Flk-1^-CD34^-CD31^+ cluster from the EPC category in this study because of their lack of differentiation potential into mature ECs in vitro.

There is accumulating evidence that a decrease in the number and functional activities of EPCs is closely linked to cardiovascular disease (13, 19, 35, 37). Changes in EPCs have actually been documented in patients with diabetes (13, 19). Hyperglycemia per se and oxidative stress are considered to actually be a center of the reticulo-endothelial system thereby promoting tissue healing (29). The spleen is thus injured tissues, turn into dendritic cells and macrophages, accumulation of splenic EPCs to increase by a factor of 2.18 (Figs. 3, 5, and 6). Moreover, diabetic conditions cause the derangement of EPCs in diabetes (8).

The present study has demonstrated for the first time the accumulation of EPCs in the spleen under healthy conditions (Figs. 3, 5, and 6). Moreover, diabetic conditions cause the accumulation of splenic EPCs to increase by a factor of 2.18 (Fig. 6) despite the absence of splenomegaly (Fig. 1). The spleen plays important roles in various vertebrate biological processes, such as removal of aged erythrocytes, recycling of iron, elicitation of immunity, and supply of thrombocytes and erythrocytes after hemorrhagic shock (29). Recently, the spleen has been found to contain in its reserve nearly half of the whole body’s monocytes (29). These monocytes, upon moving to injured tissues, turn into dendritic cells and macrophages, thereby promoting tissue healing (29). The spleen is thus considered to be a center of the reticulo-endothelial system functioning in the storage and rapid deployment of blood and immune cells. We speculate that the spleen is a major reservoir of EPCs. Possible mechanisms for the enhanced accumulation of splenic EPCs under diabetic conditions include 1) an increase in EPC proliferation or differentiation from HSC only in the spleen, 2) an increase in trapping blood EPCs, and 3) a decrease in EPC release. Further experiments are warranted to elucidate the precise mechanism involved.

We thus reveal for the first time the spatial changes in mouse EPC number in diabetes. Under diabetic conditions, the production of EPCs in the BM and the mobilization of EPCs from the spleen seem to be impaired, resulting in diminished circulating EPCs, which in turn contributes to the development and exacerbation of diabetic vascular complications.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

H.S. and Y.Y. performed the experiments; H.S. and Y.Y. analyzed the data; H.S. and Y.Y. prepared the figures; H.S. and Y.Y. drafted the manuscript; H.S., Y.Y., and H.Y. approved the final version of the manuscript; Y.Y. and H.Y. conception and design of the research; Y.Y. and H.Y. interpreted the results of the experiments; Y.Y. and H.Y. edited and revised the manuscript.

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