Endothelial Progenitor Thrombospondin-1 Mediates Diabetes-Induced Delay in Reendothelialization Following Arterial Injury

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Abstract—Delayed reendothelialization contributes to restenosis after angioplasty and stenting in diabetes. Prior data have shown that bone marrow (BM)-derived endothelial progenitor cells (EPCs) contribute to endothelial recovery after arterial injury. We investigated the hypothesis that the EPC contribution to reendothelialization may be impaired in diabetes, resulting in delayed reendothelialization. Reendothelialization was significantly reduced in diabetic mice compared with nondiabetic mice in a wire-induced carotid denudation model. The EPC contribution to neoendothelium was significantly reduced in Tie2/LacZ BM-transplanted diabetic versus nondiabetic mice. BM from diabetic and nondiabetic mice was transplanted into nondiabetic mice, revealing that reendothelialization was impaired in the recipients of diabetic BM. To examine the relative roles of denuded artery versus EPCs in diabetes, we injected diabetic and nondiabetic EPCs intravenously after arterial injury in diabetic and nondiabetic mice. Diabetic EPCs recruitment to the neoendothelium was significantly reduced, regardless of the diabetic status of the recipient mice. In vitro, diabetic EPCs exhibited decreased migration and adhesion activities. Vascular endothelial growth factor and endothelial NO synthase expressions were also significantly reduced in diabetic EPCs. Notably, thrombospondin-1 mRNA expression was significantly upregulated in diabetic EPCs, associating with the decreased EPC adhesion activity in vitro and in vivo. Reendothelialization is impaired by malfunctioning EPCs in diabetes. Diabetic EPCs have phenotypic differences involving thrombospondin-1 expression compared with nondiabetic EPCs, revealing potential novel mechanistic insights and therapeutic targets to improve reendothelialization and reduce restenosis in diabetes. **(*Circ Res*. 2006;98:697-704.)**

Key Words: diabetes mellitus ■ endothelium ■ restenosis ■ carotid arteries

Numerous clinical studies have indicated that diabetic patients have a higher incidence of restenosis after percutaneous coronary interventions compared with patients without diabetes.[1–3] Excellent results have been achieved with drug-eluting stents, with significant reductions in restenosis rates. However, drug-eluting stents themselves delay reendothelialization, and diabetic patients continue to have excessive rates of restenosis, including thrombosis that may cause acute coronary syndrome.

The loss of endothelial integrity that occurs during percutaneous coronary intervention appears to be an inciting event in the accumulation of inflammatory cells and the onset of vascular smooth muscle cell proliferation and migration that contribute to the establishment of a neointimal lesion.[4] Consistent with this hypothesis, recovery of endothelial integrity, ie, reendothelialization, has been shown to inhibit neointimal thickening. Endothelial dysfunction is also thought to be a factor that contributes to the increased incidence of restenosis in diabetes.[5] Indeed, it has been demonstrated that neointimal formation was exacerbated in diabetic animals.[6,7]

Our laboratory[8] and others[9] have shown that endothelial cells (ECs) adjacent to the site of balloon injury might not constitute the sole participants in endothelial recovery. These studies have indicated that circulating cells, referred to as endothelial progenitor cells (EPCs), derived from the bone marrow (BM) and exhibiting certain features consistent with EC identity, are capable of being recruited to sites of arterial injury and contributing to reestablishing the neoendothelium. Indeed, several lines of evidence have demonstrated that BM-derived EPCs, which are mobilized by a variety of factors including physical training,[10] statins,[11,12] estrogen,[13,14]

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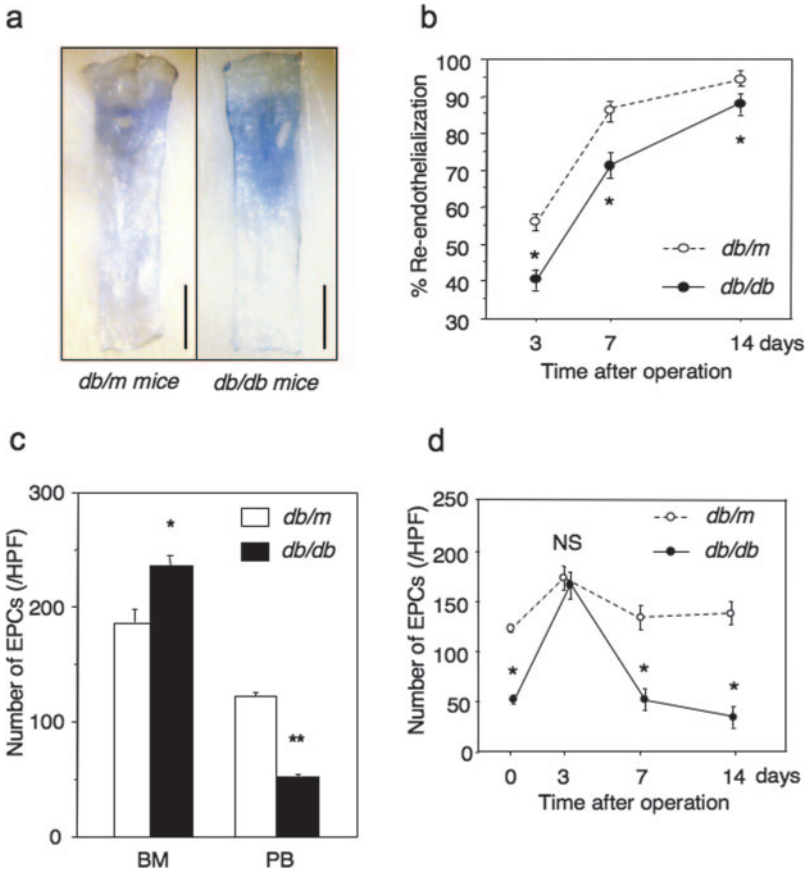

Figure 1. Reendothelialization and modulation of EPC kinetics by arterial denudation. a, Representative in situ Evans blue staining 7 days after carotid denudation in db/m (left) and db/db (right) mice. Bar=1 mm. b, Reendothelialization was quantified in Evans blue–stained en face carotid arteries 3, 7, and 14 days after operation in db/m (open circle) and db/db (closed circle) mice. *$P<0.05$ vs db/m (n=5 in each group). c, BM-derived and circulating EPCs in PB in intact db/m and db/db mice. *$P<0.05$ and **$P<0.0001$ vs db/m (n=5 in each group). d, Circulating EPC kinetics in peripheral blood after operation in db/m (open circles) and db/db (closed circles) mice. *$P<0.0001$ vs db/m (n=5 in each group).

and cytokines,[15,16] as well as exogenously infused cells,[17,18] can significantly contribute to reendothelialization after endothelial denudation following arterial injury. It has been shown that the number of circulating EPCs inversely correlates with risk factors of atherosclerosis[19,20] and that diabetes impairs the function of EPCs.[19,21,22] However, the relationship between EPC function, reendothelialization, and restenosis in diabetes has not been fully defined.

Accordingly, we performed a series of investigations to test the hypothesis that the increased incidence of restenosis in diabetes is contributed to by delayed reendothelialization resulting from phenotypic abnormalities of BM-derived EPCs.

Materials and Methods

Surgical Procedure

All procedures were approved by St. Elizabeth's Institutional Animal Care and Use Committee, consistent with the NIH *Guide for the Care and Use of Laboratory Animals*. Genetically diabetic (db/db) and heterozygous littermate control (db/m) C57BLKS/J mice of males between 6 and 8 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, Me). Wire-induced carotid denudation was performed as described previously.[13] Details are provided in the online data supplement (#1), available at http://circres.ahajournals.org.

Allogenic Bone Marrow Transplantation Model

Male db/db or db/m mice aged 4 to 6 weeks were used as recipients for BM transplantation (BMT). Transgenic mice of FVB/N-TgN [Tie2-LacZ] 182Sato, db/m, or db/db mice (The Jackson Laboratory) were used as donors for the BMT. The procedure of allogenic BMT was performed as described previously,[23] with some modifications. Details are provided in the online data supplement (#2).

EPC Culture Assay and BM-Derived EPC Culture

EPC culture assay and BM-derived EPC culture was performed as described previously.[23,24] Details are provided in the online data supplement (#3). Briefly, cultured EPCs were quantified by counting the total number in 500 μL of peripheral blood and averaged. EPCs isolated from BM were used for cell function assay, cell transfusion study, Western blot, and real-time PCR analysis. All cells for in vitro study were obtained from db/m, db/db, or thrombospondin (TSP)-$1^{-/-}$ mice.

EPC Transfusion Study

To enhance the detection of infused EPCs, splenectomy was performed in all mice 7 days before carotid denudation and EPC transfusion. The spleen was removed with vessel ligation through a lateral incision in the left abdominal wall. Cultured BM-derived EPCs were labeled with DiI-acLDL or transfected with TSP-1 plasmid DNA before transfusion to mice. Recipient mice were administered 1×10^6 of EPCs by intravenous injection from tail vein just after induction of arterial injury. Denuded carotid arteries were examined 7 days for Evans blue staining and 14 days for en face histological assessment after surgery.

Histological Assessment

To measure the reendothelialized area, animals were perfused in vivo with Evans blue dye (Sigma) 3, 7, and 14 days after surgery, as described previously.[13] Details are provided in the online data supplement (#5).

The carotid arteries from BMT mice were harvested 14 days after surgery. X-Gal staining was performed on en face denuded arteries to visualize and quantify BM-derived Tie2/LacZ–positive endothelial lineage cells, as described previously.[11,13] X-Gal–positive cells

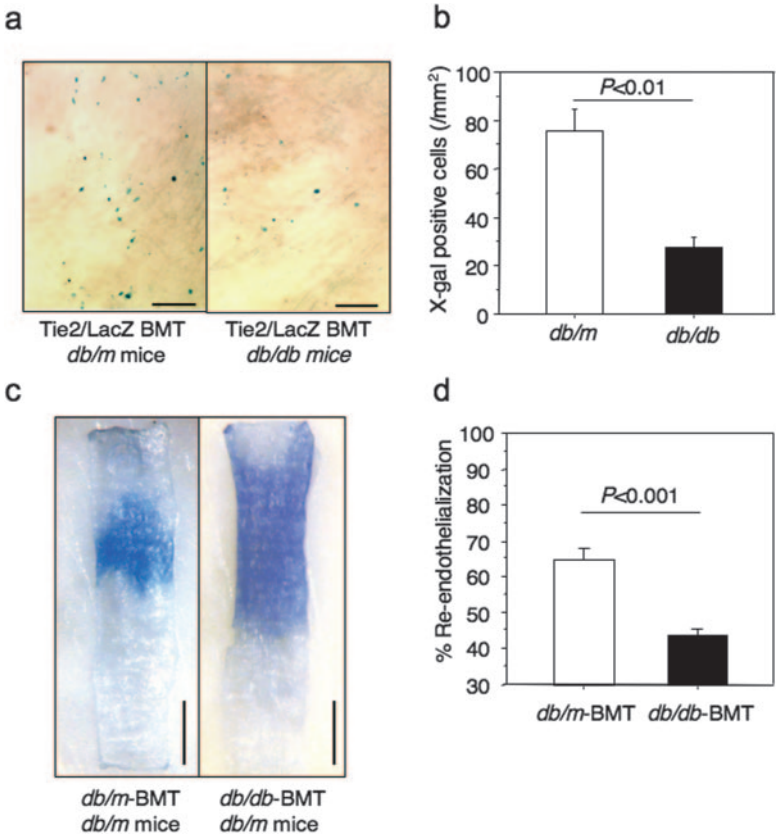

Figure 2. EPC recruitment to neoendothelium after carotid denudation. a, Representative in situ en face X-gal–staining images of denuded carotid arteries 14 days after surgery in Tie2/LacZ BMT db/m and Tie2/LacZ db/db mice. Bar=100 μm. b, Quantification of X-gal–positive cells on injured carotid arteries. db/m, Tie2/LacZ BMT db/m mice, and db/db, Tie2/LacZ BMT db/db mice (n=5 in each group). c, Representative in situ Evans blue staining 7 days after carotid denudation in db/m BMT db/m mice (left) and db/db BMT db/m mice (right). Bar=1 mm. d, Reendothelialization was quantified in Evans blue–stained en face carotid arteries 7 days after operation. db/m BMT, db/m BMT to db/m mice, and db/db BMT, db/db BMT to db/m mice (n=5 in each group).

were counted on 5 different en face carotid arteries under a light microscope and expressed as average number per surface area (in square millimeters). Exogenously administered DiI-acLDL–labeled cultured EPCs were detected in red fluorescence on en face denuded arteries 14 days after surgery. DiI-positive cells were counted under a fluorescent microscope (Nikon ECLIPSE TE200) and quantified in the same way.

EPC Function Assay: Adhesion, Migration, and Proliferation Activity

EPC adhesion to extracellular matrices was evaluated as described previously[11] with some modifications. Briefly, EPCs (2.5×10^4/well) were seeded on 96-well plates pre-coated with vitronectin, collagen type I, fibronectin and laminin (Sigma) (1 hour at 37°C) and incubated for 1 hour at 37°C and 5% CO_2. After washing 3 times with PBS, attached cells were counted. Adhesion activity was evaluated as the mean number of attached cells in one high power field (×10 or ×20) from 8 wells.

EPC migrations were evaluated using a modified Boyden's chamber assay as described previously.[23] Briefly, cell suspensions (5×10^4 cells/well) were placed in the upper chamber at the indicated glucose concentrations, and the lower chamber was filled with medium containing mouse recombinant vascular endothelial growth factor (VEGF) (50 ng/mL) (R&D Systems). The chamber was incubated for 16 hours at 37°C and 5% CO_2. Migration activity was evaluated as the mean number of migrated cells in 5 high power fields (×40) per chamber.

EPC proliferation was evaluated by bromo-deoxyuridine (BrdUrd) incorporation as described previously.[25] Briefly, cells (2.5×10^4 cells/well) were seeded on rat vitronectin (50 μg/mL at 37°C for 1 hour) coated 4-well chamber slides and cultured in regular medium with glucose following 24 hour-serum starvation. After 18 hours in culture, BrdUrd (10 μmol/L, Sigma) was added to each well and incubated with cells for 6 hours. BrdUrd was visualized by immunostaining with anti-BrdUrd antibody (1:100, Biodesign), and proliferation activity was expressed as averaged BrdUrd positive cell percentage in 5 high-power fields (×40).

Western Blot Analysis

Western blot analysis was performed as described previously.[24] Briefly, protein extracts from the cells cultured under low or high-glucose conditions were electrophoresed and immunoblotted with anti–endothelial NO synthase (eNOS) (1:1000, BD Pharmingen), phospho-eNOS (1:1000, Cell Signaling), -VEGF (1:500, Santa Cruz Biotechnology), and α-actin (1:1000, Santa Cruz Biotechnology) antibody followed by incubation with horseradish peroxidase–anti-rabbit IgG (1:5000, Santa Cruz Biotechnology). Immunoreactive bands were visualized with ECL reagent (Amersham) and were quantified densitometrically using NIH Image 1.63 software.

Quantitative Real-Time RT-PCR

Cells were lysed with RNA-Stat (Tel-Test Inc), RNA was extracted and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad), and amplification was performed on the TaqMan 7300 (Applied Biosystems) according to the instructions of the manufacturer. Primer and probe sequence are in the online data supplement (#4). Relative mRNA expression of the target gene was calculated with the comparative C_T method. The amount of the target gene was normalized to the endogenous 18S control gene (Applied Biosystems) and calculated as a relative expression=2^{DCT}. Adhesion molecule and extracellular matrix relating genes between db/m and db/db mice were screened by cDNA microarray kit (SuperArray).

TSP-1 Plasmid DNA transfection

A full-length human TSP-1 cDNA fragment (3.5 kb) was amplified by PCR, using human Quick clone placenta cDNA (Clontech) as a template, and was ligated into the *Xho*I and *Hpa*I sites of a MSCV-I-GFP retroviral expression plasmid that contains the MSCV long terminal repeat driving expression of enhanced GFP (Clontech). Cultured EPCs were transfected with human TSP-1 plasmid DNA, using transfection reagent Arrest-In (Open Biosystems) according to

the instructions of the manufacture. Cells were used for quantitative RT-PCR for mouse and human TSP-1 mRNA and adhesion assay 3 days after the transfection.

Statistical Analysis

All data are presented as mean±SEM. Significance ($P<0.05$) was determined by ANOVA followed by post hoc analysis with the Fisher procedure.

Results

Delayed Reendothelialization Is Associated With Decreased Circulating EPCs in Diabetic Mice

Figure 1a shows examples of arteries harvested 7 days after injury from db/m and db/db mice. The db/db mice exhibited delayed reendothelialization as compared with db/m mice at all time points after injury (day 3: 39.1±2.6 versus 55.9±2.3%; day 7: 69.7±3.1 versus 85.7±2.7%; day 14: 87.6±2.1 versus 94.6±1.9%; $P<0.05$, respectively) (Figure 1b).

Before carotid injury, the number of circulating EPCs was significantly reduced in db/db mice compared with db/m mice (50.8±4.1 versus 122.0±3.4/high power field [HPF]; $P<0.0001$). In contrast, the number of EPCs in the BM was significantly greater in db/db mice than in db/m mice (234.2±9.9 versus 186.2±12.3/HPF; $P<0.05$) (Figure 1c). After arterial injury, the EPC number increased, peaking at day 3 and returning to baseline by day 7 in both db/db and db/m mice. Although the peak level of EPC mobilization was similar at day 3 (165.6±14.3 versus 172.8±11.4/HPF; P=NS), the extent of decrease in the number of circulating EPCs was significantly greater in db/db mice than that in db/m mice at day 7 (52.0±10.6 versus 134.0±12.0/HPF; $P<0.0001$). The circulating EPC number was further decreased in db/db mice, resulting in a significant difference between db/db and db/m mice at day 14 (31.6±7.4 versus 144.0±10.3/HPF; $P<0.0001$) (Figure 1d).

Contribution of EPCs to Endothelial Recovery Is Reduced in Diabetes

As shown in Figure 2, whole-mount X-gal–stained carotid arteries from Tie2/LacZ BMT db/db mice displayed decreased numbers of X-gal–positive cells on the luminal surface than in Tie2/LacZ BMT db/m mice 14 days after arterial injury (27.2±4.5 versus 75.8±9.3 cells/mm^2; $P<0.01$) (Figure 2b). Next, the contribution of diabetic versus nondiabetic BM-derived cell to reendothelialization was evaluated 7 days after carotid denudation in nondiabetic mice to determine whether the diabetic phenotype of the BM cells influenced their participation in endothelial recovery (donors: db/db mice; recipients: db/m mice) versus nondiabetic BMT nondiabetic mice (donors: db/m mice; recipients: db/m mice). Evans blue staining of whole-mounted carotid arteries shows delayed reendothelialization in nondiabetic mice with diabetic BM compared with that in nondiabetic mice with nondiabetic BM (Figure 2c) (db/m-BM versus db/db-BM) (43.5±2.1 versus 64.4±2.7%; $P<0.001$) (Figure 2d). These data demonstrate that delayed reendothelialization in diabetic mice involves impaired EPC incorporation into the carotid artery neoendothelium.

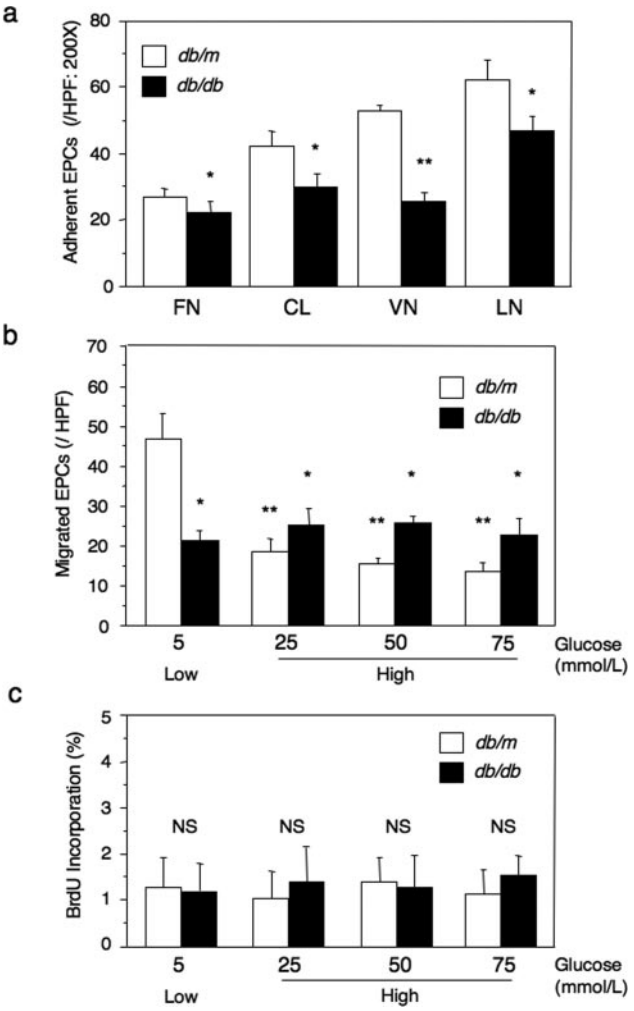

Figure 3. Cellular functions of EPCs isolated from db/m and db/db mice under high-glucose condition. a, Adhesion activity of nondiabetic (db/m) EPCs and diabetic (db/db) EPCs. *$P<0.05$, **$P<0.0001$ vs db/m. Culture dishes were coated with extracellular matrices. FN indicates fibronectin; CL, type I collagen; VN, vitronectin; LN, laminin. b, Migration activity of nondiabetic (db/m) and diabetic (db/db) EPCs under high-glucose condition. *$P<0.001$ vs db/m (Low) (5 mmol/L) and P=NS vs db/db (Low) (5 mmol/L). c, Proliferation activity assessed by BrdUrd incorporation of nondiabetic (db/m) and diabetic (db/db) EPCs under high-glucose condition. P=NS, db/m vs db/db. Similar results were obtained from 3 independent experiments.

Diabetic EPCs Exhibit Not Only Less Adhesion and Migration Activity but Also Decreased Cytokine Expressions

To further examine the phenotype of diabetic EPCs, we performed in vitro studies. The adhesion activity of diabetic EPCs was significantly reduced against all tested extracellular matrices: fibronectin, 19% reduction; collagen I, 18%; vitronectin, 51%; and laminin, 24% compared with that of nondiabetic EPCs (Figure 3a). The migration activity of diabetic EPCs was significantly reduced compared with normoglycemic-cultured, nondiabetic EPCs independent of the glucose conditions of the diabetic EPCs (5 mmol/L: 21.4±2.5; 25 mmol/L: 25.1±4.4; 50 mmol/L: 25.6±1.9; 75 mmol/L: 22.9±3.1; versus 5 mmol/L: 46.9±6.6/HPF; $P<0.001$). Nondiabetic EPCs also exhibited significantly

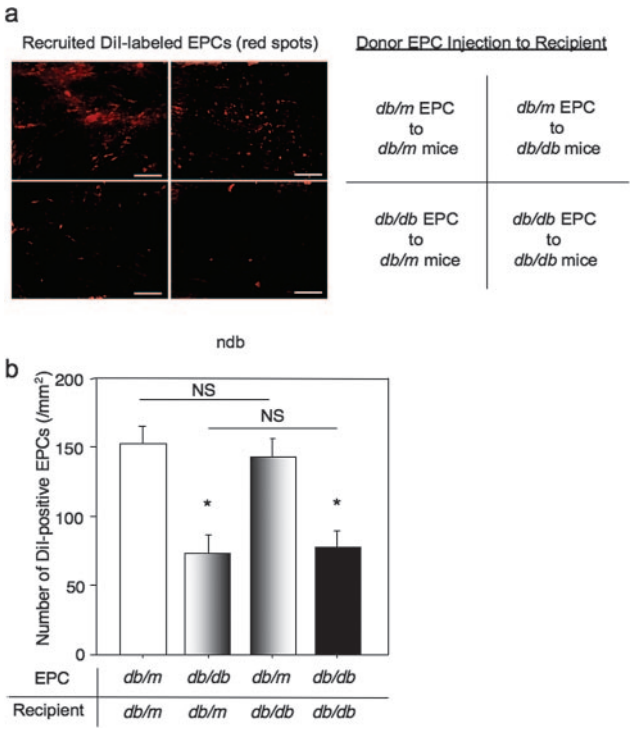

Figure 4. Exogenous EPC recruitment to neoendothelium after arterial denudation. a, Representative fluorescence images of en face carotid arteries 14 days after surgery. Red spots indicate exogenously administered DiI-labeled EPCs that recruited to neoendothelium (left panels). Bar=100 μm. Right, Phenotypes of donor EPC and recipient mice that correspond to left images are shown, respectively. b, Quantitative analysis of recruited DiI-positive EPCs on en face carotid arteries. *P<0.001 vs db/m-db/m (n=5 in each group).

reduced migration activity under high-glucose conditions (25 mmol/L: 18.8±3.1; 50 mmol/L: 15.9±1.2; 75 mmol/L: 14.4±1.7; versus 5 mmol/L: 46.9±6.6/HPF; P<0.001) (Figure 3b). On the other hand, there were no significant differences in proliferative activity (percentage of BrdUrd incorporation) between diabetic and nondiabetic EPCs, independent of glucose condition (5 mmol/L: 1.3±0.6 versus 1.2±0.6; 25 mmol/L: 1.0±0.6 versus 1.4±0.5; 50 mmol/L: 1.4±0.5 versus 1.3±0.7%; 75 mol/L: 1.2±0.6 versus 1.5±0.5; P=NS) (Figure 3c).

Relative Role of Diabetic EPCs Versus Diabetic Vessel Wall in Delayed Reendothelialization

The above experiments indicated that a BMT model would be insufficient to evaluate a nondiabetic EPCs function in a diabetic animal, because the EPC phenotype would be impaired during the 4 weeks of recovery following BMT. Accordingly, to examine the relative role of the diabetic phenotype of circulating EPCs versus the injured vascular bed in the incorporation of EPCs in the neoendothelium, we performed EPC infusion experiments. Cultured EPCs isolated from db/db or db/m mice BM were labeled with DiI and administered systemically to splenectomized db/db mice or db/m mice via tail vein immediately after carotid denudation. Diabetic EPC recruitment to the neoendothelium was markedly reduced regardless of the diabetic status of the recipient mice (db/db EPC to db/m or db/db mice; Figure 4a, lower panels). On the other hand, nondiabetic EPC recruitment to the neoendothelium was observed with similar frequency in both in nondiabetic and diabetic recipients (db/m EPC to db/m or db/db mice; Figure 4a, upper panels). Thus, the number of recruited diabetic EPCs was significantly reduced in db/m and db/db mice compared with that of recruited nondiabetic EPCs in db/m mice (73.6±12.8 and 77.4±12.9 versus 152.2±13.2 cells/mm^2; P<0.001 and P<0.001, respectively). There were no significant differences between nondiabetic and diabetic mice in the number of incorporated nondiabetic or diabetic EPCs (152.2±13.2 versus 143.4±12.8 and 73.6±12.8 versus 77.4±12.9 cells/mm^2; P=NS and P=NS, respectively) (Figure 4b). These data, while being interpreted cautiously with the knowledge that a cultured EPC was used, further support the above findings, indicating a reduced contribution of diabetic EPCs to reendothelialization that is independent of the diabetic or nondiabetic environment of the injured vascular beds.

Diabetic EPCs Exhibit Altered Expression of NOS, VEGF, and TSP-1

Next, we examined the expression of certain candidate genes in diabetic and nondiabetic EPCs under varying glucose conditions. The expression of phosphorylated form of eNOS was significantly reduced in diabetic EPCs even at the lowest glucose concentration (relative expression in arbitrary units 0.20±0.06; P<0.0001, respectively). In contrast, eNOS and phospho-eNOS expression was reduced in nondiabetic EPCs only at higher glucose concentrations (Figure 5a). Similarly, the expression of VEGF was also significantly decreased in diabetic EPCs at lower glucose concentrations, whereas in nondiabetic EPCs VEGF expression was reduced in a glucose concentration–dependent manner (Figure 5b). cDNA microarray analysis (supplemental Figure I) indicated that the expression of TSP-1 was upregulated in diabetic EPCs. The regulation of TSP-1 was further confirmed by quantitative real-time PCR. TSP-1 mRNA expression was significantly increased in cultured EPCs under high-glucose condition in a concentration-dependent manner (25 mmol/L: 3.2±0.4; 50 mmol/L: 4.2±0.8; 75 mmol/L: 3.8±0.3; versus 5 mmol/L: 1.2±0.2; P<0.0001). Although the expression of TSP-1 in db/db EPCs was similar to that in db/m EPCs under low-glucose conditions, it was also upregulated by high glucose in a dose-dependent manner (data not shown). TSP-1$^{-/-}$ EPCs were included as a negative control.

TSP-1 Mediates Decreased EPC Adhesion Activity Under High-Glucose Conditions and Delayed Reendothelialization

Next, to determine the role of TSP-1 expression on EPC adhesion activity, we transfected EPCs with a TSP-1 expression vector or control vector and repeated the vitronectin adhesion experiments (Figure 4a). TSP-1 mRNA was overexpressed by TSP-1 plasmid DNA transfection in db/m EPCs under low-glucose condition over the level as under high-glucose conditions (5.2±0.3 versus [5 mmol/L] 1.2±0.2; P<0.0001). The empty vector (EV) of TSP-1 plasmid DNA transfection did not affect endogenous TSP-1 mRNA expression (1.1±0.2 versus [5 mmol/L] 1.2±0.2; P=NS) (Figure

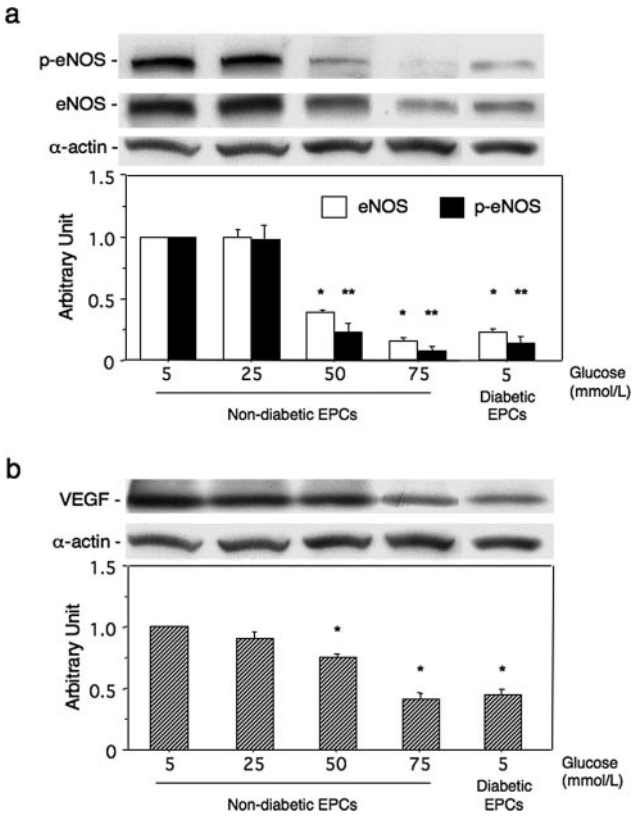

Figure 5. Western blot analyses for eNOS and VEGF expression in EPCs under high-glucose conditions. a, Representative immunoblots for eNOS and phosphorylated eNOS (p-eNOS) and quantification by densitometric analysis. eNOS and α-actin were used as loading controls for p-eNOS and eNOS expression, respectively. Assays were triplicated and averaged. *$P<0.0001$ and **$P<0.0001$ vs p-eNOS and eNOS under low-glucose (5 mmol/L) condition in nondiabetic EPCs, respectively. b, Representative immunoblots for VEGF and quantification by densitometric analysis. α-Actin was used as a loading control for VEGF expression. *$P<0.01$, low-glucose (5 mmol/L) condition in nondiabetic EPCs.

6a). Adhesion activity was now inversely related to TSP-1 expression, regardless of glucose conditions. Namely, there were no significant differences in the number of adherent cell EPCs among low-glucose conditioned db/m EPCs, medium-glucose cultured EPCs transfected with EV, or TSP-$1^{-/-}$ EPCs cultured at doses of 5, 25, and 50 mmol/L glucose (EV: 214±14.6; TSP-$1^{-/-}$: 5 mmol/L [201±22.2], 25 mmol/L [216±11.0], 50 mmol/L [226±16.6]; versus db/m: 5 mmol/L [239±11.3/HPF]; P=NS). A significant decrease of adherent cell numbers in TSP-$1^{-/-}$ EPCs was observed at a supraphysiological glucose concentration of 75 mmol/L (149±12.9 versus db/m [5 mmol/L] 239±11.3; $P<0.05$), indicating that non–TSP-1–mediated changes in adhesion activity can occur at very high-glucose concentrations. On the other hand, db/m EPCs cultured under high-glucose conditions and TSP-1–overexpressing db/m EPCs had significantly less adhesion activity compared with low-glucose conditioned db/m EPCs (25 mmol/L: 106±5.7; 50 mmol/L: 100±8.9; 75 mmol/L: 90±4.7; versus 5 mmol/L: 239±11.3; $P<0.0001$). Indeed, reendothelialization in TSP-1–overexpressing EPC-transfused nondiabetic mice was significantly slower than that in control EPC transfused nondiabetic mice (77.4±4.5 versus 89.9±1.3%; $P<0.05$) (Figure 6c and d), suggesting that TSP-1 plays a critical role in adhesion activity and may provide a mechanism by which diabetes and hyperglycemia leads to impaired reendothelialization under certain circumstances.

Discussion

The present study provides evidence that mobilization, recruitment, and incorporation of EPCs to sites of arterial injury is reduced in diabetes and contributes to the delay in reendothelialization and, by inference, to the increase in restenosis rates observed in diabetics. This observation is shown to be dependent on the diabetic EPC phenotype as well as the high-glucose environment, as both EPC adhesion and migration activity are diminished under high-glucose conditions. Moreover, high glucose reduced EPC expression of major cytokines, eNOS and VEGF, as well as the expression of 1 of the adhesion molecules, TSP-1. These findings are consistent with recent publications[21,22] and add the potentially important finding of a TSP-1–dependent mechanism for the affects of high glucose/diabetes on EPC adhesion and migration activity. Notably, the present findings suggest that reduced EPC recruitment to sites of arterial injury may be a central feature in the pathophysiology of the diabetic response to vascular injury. It has long been thought that endothelial regeneration in response to arterial injury was a local process involving endothelial proliferation and migration from intact ECs adjacent to the site of injury. The present data suggest that delayed reendothelialization after injury in diabetes is a significant byproduct of failed EPC biology. However, because EPCs are not originated from single cell type but so-called "heterogenous cell population," we have to keep in our mind that certain subpopulations in the heterogenous EPCs may act differently in response to vascular injury as a study limitation.

The present data show that the number of circulating EPCs in diabetic mice is significantly reduced before and after (days 7 and 14) arterial injury compared with that in nondiabetic mice; however, the peak EPC number reached to similar level in both diabetic and nondiabetic mice, suggesting that the retention of circulating EPCs rather than the mobilization from BM is impaired in diabetes. Indeed, the number of EPCs in BM in diabetic mice was greater than that in nondiabetic mice. One of the possible reasons for this decreased number of EPCs in peripheral blood in diabetic mice is considered to be attributable to reduced expression of certain cytokines, such as VEGF, which can maintain EPCs in the circulation.[26]

By using various BMT models and exogenous cultured EPC transfusion with carotid denudation, we were able to demonstrate that diabetic EPCs have less potential to be recruited to the neoendothelium. Because nondiabetic EPC recruitment does not account for the majority of ECs repopulating the neoendothelium, one must consider paracrine factors, both positive and negative, that might influence endothelial recovery differentially in diabetics and nondiabetics. Specifically it is possible that diabetic BM-derived cells, including EPCs, may actually act to delay reendotheli-

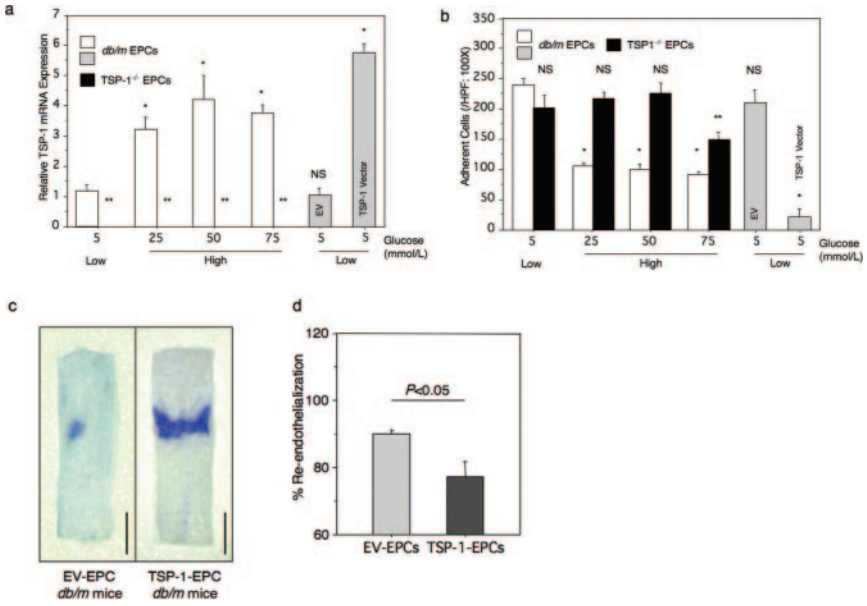

Figure 6. TSP-1 mRNA expression and effect on adhesion activity in EPCs. a, TSP-1 mRNA expression in nondiabetic (db/m) and TSP-1$^{-/-}$ EPCs under high-glucose condition for 24 hours. EPCs were transfected with TSP-1 plasmid DNA (TSP-1 Vector) or its backbone empty vector (EV) and incubated for 48 hours. Assays were triplicated and averaged. *$P<0.0001$ and **P=NS vs db/m (Low) (5 mmol/L). b, Adhesion activity to vitronectin of nondiabetic (db/m), empty vector (EV), transfected and TSP-1 plasmid DNA (TSP-1 Vector) transfected EPCs. EPCs were cultured under high-glucose conditions for 3 days before the assay. *$P<0.0001$, **$P<0.05$ and P=NS vs db/m (Low) (5 mmol/L). Similar results were obtained from 3 independent experiments. c, Representative in situ Evans blue staining 7 days after carotid denudation in the empty vector (EV) transfected EPC transfusion group (EV-EPC) and in the TSP-1–overexpressing EPC transfusion group (TSP-1-EPC). Bar=1 mm. d, Reendothelialization was quantified in Evans blue–stained en face carotid arteries 7 days after operation (n=5 in each group).

alization (Figure 2), an effect beyond the decreased BM-derived EPC contribution, which contributes to slower reendothelialization. Furthermore, the diabetic phenotype of EPCs not only leads to a decreased contribution to the neoendothelium but also appears to have a decreased effect on preexisting mature ECs, which are also major contributors in reendothelialization, because of decreased production of VEGF and eNOS (Figure 5). From the above observations, one must consider that EPCs play a role as paracrine effectors as well as direct contributors in reendothelialization.

A critical initial event for reendothelialization, in terms of EPC contribution, is considered to be the attachment of circulating EPCs to the denuded arterial wall; therefore, the modulation of EPC adhesion activity in diabetes would be a key consideration. Our data reveal the upregulation of EPC TSP-1 expression under high-glucose conditions. The upregulation of TSP-1 in vascular cells including ECs, SMCs, and fibroblasts in diabetic rats has recently been reported[27]; however, until now, no direct evidence for TSP-1 upregulation has shown in diabetic EPCs. Furthermore, we also demonstrated the inverse association between TSP-1 expression and adhesion activity in EPCs, suggesting that TSP-1 would be, at least in part, a key adhesion molecule to extracellular matrices and that high glucose mediates impaired EPC adhesion activity via TSP-1 upregulation in diabetic EPCs. Indeed, recent accumulating evidence shows that TSP-1 is a multifunctional protein that interacts with a number of matrix proteins and cell surface receptors and may represent an important link between diabetes and high-glucose levels and the accelerated development of atherosclerotic lesions in diabetes.[27–30] TSP-1 is known to have a specific binding site to fibronectin; therefore, mature ECs have high affinity to fibronectin/TSP-1 complex via $\alpha_3\beta_1$ integrin, which is on the EC cell surface.[31] This may explain, in part, why the difference of EPC adhesion to fibronectin between EPCs of nondiabetic and diabetic mice was more modest compared with vitronectin (Figure 4a). The full implications of these findings for our understanding of the role of TSP-1 on vascular biology in diabetes remain to be further investigated.

In the present study, we used a wire-induced carotid denudation model in which the medial injury is limited for evaluating reendothelialization. Although this animal model may not be suitable for evaluating vascular smooth muscle cells, which have potential effects and exceed the effects on endothelium in diabetes, the present study reveals that delayed reendothelialization in diabetes is mediated, in part, by deficits in BM-derived EPC contribution to sites of neoendothelium in injured arteries. This may be the result, in part, of reduced numbers of circulating EPCs, as has been shown in human diabetics,[22] but is also clearly a phenotypic feature of the diabetic EPCs themselves, as documented in the infusion studies. The reduced contribution of EPCs appears to be attributable to the impaired adhesion activity associating with upregulation of TSP-1 expression; however, the precise mechanism by which TSP-1 reduces EPC adhesion activity remains to be determined. In addition, our data simultaneously suggest that EPCs may provide a major contribution in the nature of endothelial recovery after arterial injury.

In conclusion, these data provide novel insights into potential mechanisms of delayed reendothelialization in diabetes, demonstrating a potentially important role for TSP-1 in regulating diabetic EPC biology. Modulation of TSP-1 expression in EPCs could represent a novel therapeutic approach for preventing restenosis after arterial intervention in diabetic patients.

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