Circulating endothelial progenitor cells and cellular membrane microparticles in db/db diabetic mouse: possible implications in cerebral ischemic damage

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Chen J, Chen S, Chen Y, Zhang C, Wang J, Zhang W, Liu G, Zhao B, Chen Y. Circulating endothelial progenitor cells and cellular membrane microparticles in db/db diabetic mouse: possible implications in cerebral ischemic damage. Am J Physiol Endocrinol Metab 301: E62–E71, 2011. First published April 19, 2011; doi:10.1152/ajpendo.00026.2011.—For determining the implication of circulating endothelial progenitor cells (cEPCs) and cellular membrane microparticles (MPs) in diabetic stroke, levels of EPCs, EPC-MPs, and endothelium-derived MPs (EMPs) and their correlations with blood glucose concentration, cerebral microvascular density (cMVD), and ischemic damage were investigated in type 2 diabetic db/db and db/+ (wild-type control) mice. Therapeutic efficacy of EPC infusion (preincubated with MPs) was also explored. Ischemic stroke was induced by middle cerebral artery occlusion (MCAO) surgery. Ischemic damage and cMVD were determined using histological analyses. The levels of cEPCs and MPs were determined using flow cytometric analyses. EPC generation and function were evaluated by in vitro cell cultures. Results showed the following. 1) In db/db mice, the basal level of cEPCs was less and cMVDs were lower, but the levels of translating EPC-MPs and EMPs were more; 2) MCAO induced a larger infarct volume and less of an increase in cEPCs in db/db mice; 3) the level of cEPCs correlated with blood glucose concentration (negatively), cMVD (positively), and ischemic damage (negatively), but the levels of EPC-MPs and EMPs correlated inversely with those parameters; 4) EPCs were reduced and dysfunctional in db/db mice, and preincubation with db/db MPs impaired EPC functions; and 5) infusion of EPC-MPs preincubated with db/+ MPs increased the level of cEPCs and reduced ischemic damage, and these beneficial effects were reduced or lost in EPCs preincubated with db/db MPs. These data suggest that reduced cEPCs, impaired EPC generation/function, and increased production of MPs might be the mechanisms responsible for increased ischemic damage seen in db/db mice.

IN DIABETIC PATIENTS, ISCHEMIC CEREBRAL DAMAGE is exacerbated, and the outcome is poor. The mechanisms responsible are not well understood. Vascular rarefaction and reduced collateralization because of impaired angiogenesis could be a possible mechanism. This is suggested from previous studies on peripheral vascular complications (21, 23) and wound healing (7, 41) in diabetes. However, whether this mechanism is also applicable for ischemic stroke in diabetes has not been investigated.

Circulating endothelial progenitor cells (cEPCs), defined as bone marrow (BM)-derived circulating immature cells with the ability to differentiate into mature endothelial cells (ECs), are known to participate in vascular homeostasis and angiogenesis (5, 46, 57). There is accumulating evidence that cEPCs are reduced and are dysfunctional in diabetes. Possible mechanisms may include decreased generation, reduced mobilization, and shortened survival (21, 23, 52). Hyperglycemia can directly impair in vitro proliferation, colony formation, migration, and tube formation as well as increase senescence of EPCs (13, 22). The cEPCs are reduced by 53% in number and show reduced clonogenic and adhesion capacity in diabetic patients with peripheral artery disease (23). Thus, alterations in EPC number and function may have an important role in the pathogenesis and progression of diabetic vascular complications. However, there is less information regarding the correlation of cEPCs with cerebral vascular density (as an index of angiogenesis) and ischemic injury.

Cellular membrane microparticles (MPs) are cell membrane fragments shed upon activation or apoptosis, with sizes ranging from 0.1 to 1 μm (45, 47). Elevated levels of circulating MPs have been reported as potential biomarkers for various cardiovascular diseases, such as acute coronary syndrome, hypertension, and ischemic stroke (19, 32, 37, 42). More interestingly, circulating MPs might represent a novel network for intercellular communication (45). It has been shown that circulating MPs isolated from patients with myocardial infarction impair endothelium-dependent vasorelaxation, whereas MPs from nonischemic patients had no effect on vasomotor function (9). Circulating MPs can be generated from ECs [endothelium-derived MPs (EMPs)] (37) as well as from EPCs (EPC-MPs) (48). Elevated levels of EMPs have been described in cerebrovascular diseases (37) and acute coronary syndrome (33). Moreover, circulating EMPs could be a biomarker of EC injury and the pathogenesis of cardiovascular diseases (14). Circulating EPC-MPs could be an indicator for EPC incompetence (48). Thus, circulating EMPs and EPC-MPs would serve as integrative biomarkers for vascular complications in diabetes.

In this study, we comprehensively analyzed the levels of cEPCs, EPC-MPs, and EMPs in db/db type 2 diabetic mice and their wild-type control (db+) mice at basal and following ischemic stroke. Their correlations with fasting blood glucose, cerebral microvascular density (cMVD), and ischemic damage were determined. To further explore the possible role of circulating MP and cEPC alterations in ischemic cerebral injury in diabetes, we evaluated the effect of MPs on EPC functions in cell cultures as well as the influence on EPC-based therapy in experimental ischemic stroke.
METHODS

**Experimental animals.** Male C57BL/6J diabetic db/db mice (8–10 wk) and their age-matched controls (db/+ ) were bought from Jackson Laboratories (Bar Harbor, ME). The level of fasting plasma glucose was determined using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostics, Indianapolis, IN). All experimental procedures were approved by the Wright State University Laboratory Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (NIH).

**Middle cerebral artery occlusion for inducing focal ischemic stroke and quantification of infarct size.** The db/db and db/+ mice (n = 12/group) were randomly divided into two subgroups for middle cerebral artery occlusion (MCAO) or sham surgery (n = 6/subgroup). MCAO surgery was performed to induce focal cerebral ischemic stroke, as described previously (11). For MCAO sham surgery, the suture was immediately drawn back by 1–2 mm after the opening of the middle cerebral artery was achieved. Mice were euthanized 48 h after surgery. The brains were immediately collected and fixed in 4% paraformaldehyde (PFA) overnight and in 4% PFA plus 30% sucrose for 3 days. The brains were then cut into coronal sections (20 μm) and sequentially put into six separate wells of a 24-well plate containing 2 ml of phosphate-buffered saline (PBS). Cerebral ischemic damage was revealed by Fluoro-Jade (Histochem, Jefferson, AR) staining, as described previously (20). Briefly, one well of brain sections was mounted and air-dried on microscope slides. Slides were placed in decreasing concentrations (100, 95, 85, and 75%) of ethanol for 3 min, in distilled deionized water for 1 min, and in 0.06% KMNO4 for 15 min. Brain sections were stained with 0.001% Fluoro-Jade in 0.1% acetic acid for 30 min at room temperature (RT) in the dark. Then slides were rinsed with ddH2O four times for 1 min each, dried for 20 min, cleared in xylene, and coverslipped with DPX mounting medium (BDH Laboratories, Poole, UK). The ratio of infarct volume was calculated using Image J software (NIH), as we described previously (11).

**Measurement of cMVD in nonischemic cerebral cortex and in peri-infarct area.** Measurement of cMVD in brain was achieved by immunofluorescence staining, as described previously, with slight modification (20, 30). Briefly, the free-floating brain sections were blocked for 1 h with 1% donkey serum in Tris-buffered saline. Sections were then reacted with rat anti-mouse CD31 (1:50 in 1% donkey serum; BD Biosciences, San Jose, CA) primary antibody or PBS (for negative control) at 4°C overnight. Sections were incubated with Alexa Fluor 594-conjugated donkey anti-rat IgG (1:200, Molecular Probes; Invitrogen, Carlsbad, CA) secondary antibody for 30 min at RT. Images were captured with a confocal microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). Five images in the nonischemic cortex or peri-infarct regions, determined by staining the adjacent sections with Fluoro-Jade as described above, were taken from each section. Quantification of vessel density was obtained from these pictures using Image J software (NIH). The mean volume of cMVD from six sequential brain sections of individual mouse was calculated and expressed as numbers/mm².

**Isolation of mononuclear cells from peripheral blood and bone marrow.** The mononuclear cells (MNCs) were isolated from peripheral blood by gradient density centrifugation, as described previously, with slight modification (48). A volume of 0.5–1 ml of peripheral blood was taken from the left ventricle of heart into a syringe containing 1% volume of heparin (1.000 U/ml; Sigma, Fairfax, VA). Blood was diluted in 2 ml of PBS and then gently layered over 2 ml of gradient medium (Hisopaque-1083; Sigma) for centrifugation (800 g, 30 min at 4°C). The MNCs in the interface layer were transferred to a new tube, washed with PBS buffer by centrifugation at 400 g for 5 min at 4°C, and then resuspended with 100 μl PBS for flow cytometric analysis of EPCs. The upper layer was collected for MP isolation.

Isolation of MNCs from BM flushed out from tibia and femurs was similar to the gradient density centrifugation method used for peripheral blood MNCs (39). The BM MNCs were used for EPC culture, as described below.

**Isolation of circulating MPs.** After gradient density centrifuge for isolation of MNCs, the upper layer was collected and centrifuged (3,000 g, 15 min) at 10°C to obtain platelet-poor plasma (PPP). Circulating MPs in the PPP were isolated as described previously by others (8, 47). Briefly, PPP was centrifuged at 20,000 g for 45 min at 10°C to pellet MPs. The MP pellet was resuspended in 100 μl PBS, fixed with 1% PFA, and then stored at 4°C for flow cytometric analysis within 24 h.

**Flow cytometric analysis of EPCs, EPC-MPs, and EMPs.** For analysis of EPCs, freshly isolated MNCs were incubated for 30 min at 4°C in the dark with 10 μl of phycoerythrin (PE)-conjugated anti-mouse CD34 (AbD Serotec, Raleigh, NC), 5 μl of PE-Cy7-conjugated anti-mouse VEGFR (BD Biosciences), and 1 μl FITC-conjugated anti-mouse CD45 (eBioscience, San Diego, CA) antibodies. Isotype-matched (IgG) nonspecific antibodies served as negative controls. After incubation, labeled EPCs were washed with PBS three times and resuspended with 100 μl of PBS for flow cytometric analysis (Accuri C6 flow cytometer; Accuri Cytometers, Ann Arbor, MI). EPCs were defined as CD34+VEGFR+ cells in the gate of CD45+ cells, according to previous reports (48, 55, 56). The flow cytometer was set to acquire 100,000 events/sample, and analyses were performed within the CD45+ gate. The number of cEPCs was described as the number of cells per microliter of whole blood.

**Analysis of EPC-MPs.** Samples were incubated with antibodies (CD34 and VEGF-R) under the same conditions as those for EPCs, EMPs have been shown to express CD34+VEGFR+ cells in the gate of CD45+ cells, and other EC-related surface markers and can be quantified by flow cytometry (4, 33). For defining the EMPs, samples were stained with 5 μl of PE-conjugated anti-mouse CD144 antibody (BD Biosciences). The size of particles was calibrated using 1 and 2 μm flow cytometry beads (Molecular Probes; Invitrogen, Eugene, OR). MPs were defined as particles with a diameter <1.5 μm. The numbers of circulating EPC-MPs and EMPs were determined as CD34+VEGFR+ and CD144+ events, respectively, in the gate of MPs. The data were described as the number of MPs per microliter of whole blood.

**BM EPC culture.** The ability of EPC generation from BM MNCs was studied as described previously (29). Briefly, BM MNCs isolated from db/db or db/+ mice were counted and plated (1 x 10⁶ cells/well) on fibronectin-coated 24-well plates (BD Biosciences) and then grown in EC basal medium-2 supplemented with 5% FCS containing EPC growth cytokine cocktails (Lonza, Walkersville, MD). After 3 days in culture, nonadherent cells were removed by washing with PBS. Thereafter, culture medium was changed every 2 days, and cells were cultured continuously for EPC characterization, migration assay, tube formation analysis, and MP incubation studies.

**EPC characterization and generation assays.** The EPCs in the cultures were identified as adherent cells that were double-stained for Di-LDL uptake and the binding of B-lectin. In brief, the adherent cells were incubated with PE-labeled Di-LDL (Biomedical Technologies, Stoughton, MA) for 2 h at 37°C. After that, cells were fixed with 2% PFA and then counterstained with FITC-labeled B-lectin (Sigma). Cells were viewed under an inverted fluorescent microscope (Nikon, Japan). The numbers of Di-LDL and B-lectin double-stained cells in 10 different fields (magnification ×200) were counted and averaged as a measurement of EPC generation.

**EPC migration assay.** EPC migration function was evaluated using the Boyden chamber method (54). After 10 days of EPC culture, EPCs (2 x 10⁶ cells) were placed into the upper compartment of the Boyden chamber (Chemicon, Rosemont, IL) with 50 ng/ml VEGF and 100 ng/ml stromal cell-derived factor-1 (SDF-1) in the lower compartment. After 24 h, the EPCs that migrated across the membrane were counted under an inverted light microscope (Nikon), quantified, and

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higher in db/db mouse group than those in db/+ mice (P < 0.01). These data confirmed the characteristics of db/db diabetic mice used for this study. There was no difference in age between the animal groups.

Cerebral ischemic damage is increased in db/db mice. MCAO-induced focal ischemic stroke was revealed by Fluoro-J staining on PFA-fixed brain sections. Ischemic damage was detected in the cortex, stratum and hippocampus of brain (Fig. 1A). The infarct volume was ~50% larger in db/db mice compared with control animals (35.2 ± 1.6 and 23.2 ± 2.0%, db/db vs. db/+; n = 6/group, P < 0.01; Fig. 1B).

CMVDs in the nonischemic cortex and peri-infarct area are decreased in db/db mice. The cerebral microvasculature in the nonischemic cortex (Fig. 2, A and B) and peri-infarct area (Fig. 2, C and D) were revealed by CD31 staining, and the negative controls (absence of CD31 primary antibody) did not display a discernible fluorescence signal (Fig. 2, E and F). Results showed that the CMVDs were significantly less for db/db mice in the nonischemic cortex (338 ± 29 and 416 ± 32 numbers/mm², db/db vs. db/+, n = 6/group, P < 0.01; Fig. 2G) and in the peri-infarct area (165 ± 17 and 230 ± 20 numbers/mm², db/db vs. db/+; n = 6/group, P < 0.01; Fig. 2G) compared with db/+ mice.

The levels of cEPCs are reduced, and the levels of circulating EPC-MPs and EMPs are increased in db/db mice. As potential indexes for EPC and EC damage, we also measured circulating EPC-MPs and EMPs in db/db and db/+ mice. Basal

![Image](http://ajpendo.physiology.org/)

**Table 1. General characteristics of db/+ and db/db mice**

<table>
<thead>
<tr>
<th>Variants</th>
<th>db/+</th>
<th>db/db</th>
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<tbody>
<tr>
<td>Age, wk</td>
<td>8.8 ± 0.5</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>BW, g</td>
<td>28.7 ± 0.4</td>
<td>45.6 ± 0.9**</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>118.3 ± 12.5</td>
<td>528.1 ± 10.4**</td>
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Values are means ± SE; n = 12/group. BW, body weight. **P < 0.01 compared with db/+.

EPC tube formation assay. There are two types of EPCs in culture, early- and late-outgrowth EPCs (35, 40). Early-outgrowth EPCs are obtained for 7 days, and late-outgrowth EPCs emerge 2–4 wk after the start of MNC culture. For evaluating tube formation ability, EPCs were cultured for 18 days before tube formation assay because the late EPCs were considered to play a more important role in angiogenesis (12, 13). EPC tube formation assay was performed with a tube formation assay kit (Chemicon) according to the previous reports (12, 13). Briefly, ECMatrix solution was thawed on ice overnight, mixed with 10X ECMatrix diluents, and placed in a 96-well tissue culture plate at 37°C for 1 h to allow the matrix solution to solidify. EPCs were replated (1 × 10⁴ cells/well) on top of the solidified matrix solution. Cells were incubated at 37°C for 24 h. Tube formation was evaluated with an inverted light microscope. Tubes were quantified by counting sprouting microcapillary-like structures exhibiting lengths four times their width. Five independent fields were assessed for each well, and the average number of tubes per field (magnification ×200) was determined.

Incubation of EPCs with isolated circulating MPs. EPCs were prepared, and circulating MPs were isolated as described above. Before incubation, isolated circulating MPs from db/+ (db/+ MPs) or db/db mice (db/db MPs) were resuspended with EPC culture medium with a volume (0.5–1 ml) equal to the original acquired blood and served as MP incubation working buffer. Then, EPCs (1 × 10⁵ cells/well) from db/+ mice were incubated with vehicle (culture medium), db/+ MP, or db/db MP incubation buffer for a certain period (1, 12, or 48 h) at 37°C before in vitro functional assays (migration and tube formation) or in vivo infusion study.

Tail vein injection of MP-preincubated EPCs. After 48 h of incubation with MPs, incubation buffer was removed, and EPCs (from db/+ mice) were gently detached by 0.05% trypsin with EDTA at 37°C, washed with PBS, and resuspended to obtain a required cell concentration. The db/db mice were divided into three groups (n = 6/group) for EPC (2 × 10⁵ cells/100 μl) infusion: vehicle (PBS), db/+ MP-preincubated EPCs, and db/db MP-preincubated EPCs. Before tail vein injection of EPCs, mice were put under a heat lamp (~30 min) to increase blood flow to the tail vein and then restrained in a holding device (AIMS restrainer). Tail vein injection of EPCs or vehicle was performed 2 h after MCAO surgery using a 1-ml syringe with a 26-gauge ½ needle, as described previously (26). All mice were euthanized 48 h after MCAO.

Statistical analysis. All descriptive data were expressed as means ± SD for continuous variables. Comparisons for two groups were performed by Student’s t-test. Multiple comparisons were performed by one- or two-way ANOVA followed by Bonferroni’s test. The correlations of the levels of cEPCs, EPC-MPs, and EMPs with fasting blood glucose, cMVD, and infarct volume were analyzed using the Spearman’s rank correlation test (SPSS version 13.0; SPSS, Chicago, IL). For all tests, a P value <0.05 was considered significant.

RESULTS

Animal models. General characteristics of db/db and db/+ mice were summarized in Table 1. The concentrations of fasting blood glucose and body weight were significantly...
levels of cEPCs, EMPs, and EPC-MPs in db/db and db/+ mice were represented in the groups with MCAO sham surgery. The EPCs were identified as CD34+/VEGFR+ (Fig. 3, C–F) cells at the P1 gate, which were verified as CD45+ cells (Fig. 3, A and B). Similarly, the EPC-MPs and EMPs were defined as CD34+/VEGFR+ (Fig. 4, C–F) and CD144+ (Fig. 4, H–K) cellular MPs, respectively, at gate P1 with size <1.5 μm (Fig. 4A). Isotype antibodies were used as negative controls (Fig. 4, B and H–K). Flow analysis revealed that there was a 65% decrease in cEPCs (0.17 ± 0.019 and 0.48 ± 0.05 numbers/μl, db/db sham vs. db/+ sham, n = 6/group, P < 0.01; Fig. 3G) and a 150% increase in circulating EPC-MPs (1.48 ± 0.13 and 0.59 ± 0.03 numbers/μl, db/db sham vs. db/+ sham, n = 6/group, P < 0.01; Fig. 4G) in db/db mice at basal. The basal level of circulating EMPs was much increased in db/db mice compared with db/+ control animals (84.1 ± 0.2 and 17.3 ± 0.2 numbers/μl, db/db sham vs. db/+ sham, n = 6/group, P < 0.01; Fig. 4L).

Changes in the levels of cEPCs, EPC-MPs, and EMPs after ischemic stroke. After ischemic stroke (48 h after MCAO), the levels of cEPCs were increased in both db/db and db/+ mice 1.8- and 3.6-fold, respectively (P < 0.01 vs. sham, Fig. 2). Microvascular densities in the nonischemia cortex and peri-infarct area are reduced in db/db mice. Representative pictures of immunofluorescence staining (anti-CD31) for analysis of microvascular density in db/db (B and D) and db/+ mice (A and C). A and B: microvascular density in the nonischemic cortex. C and D: microvascular density in the peri-infarct area. E and F: negative control for CD31 staining. Scale bar, 50 μm. G: summarized data. *P < 0.01 vs. db/+.
EPCs were cultured from the BM of db/+ mice (wild-type controls). EPCs were preincubated with vehicle, or with the indicated reagents for 30 min before migration assays. a As expected, the magnitude of the increase in cEPCs was much lower (~8-fold) in db/db mice (P < 0.01, db/db MCAO vs. db/+ MCAO, n = 6/group; Fig. 3G). Similarly, the levels of circulating EPC-MPs were increased over the basal in both db/db and db/+ mice (P < 0.01, MCAO vs. sham, n = 6/group; Fig. 4G). Of interest, the level of circulating EPC-MPs remained higher in db/db mice (P < 0.01, db/db MCAO vs. db/+ MCAO, n = 6/group; Fig. 4G). The levels of circulating EMPs were not changed significantly in either db/db or db/+ mice (P > 0.05, MCAO vs. sham, n = 6/group; Fig. 4L).

BM-derived EPCs are reduced and dysfunctional in db/db mice. Immunofluorescence images showed that the generation of EPCs from BM was reduced in the db/db diabetic mice (38.3 ± 3.2 and 59.1 ± 5.6 cells/field, db/db vs. db/+, n = 6/group, P < 0.01; Fig. 5, A and B). The EPC migration ability was impaired in db/db mice (46.3 ± 4.6 vs. 69.1 ± 5.5 cells/field, db/db vs. db/+, n = 6/group, P < 0.01; Fig. 5C). The tube formation ability was also decreased in db/db mice (14.9 ± 2.5 vs. 34.8 ± 3.2 tubes/field, db/db vs. db/+, n = 6/group, P < 0.01; Fig. 5, A and D).

Correlations of cEPCs, EPC-MPs, and EMPs with fasting blood glucose, cMVD, and ischemic damage. The level of cEPCs was negatively correlated with fasting blood glucose level (r = -0.74, P = 0.010; Fig. 6A) and cerebral infarct volume (r = -0.88, P = 0.003; Fig. 6B) but positively associated with cMVD in the nonischemic cortex (r = 0.91, P = 0.000; Fig. 6C) and in the peri-infarct area (r = 0.87, P = 0.006; Fig. 6D). In contrast, the level of circulating EPC-MPs was positively correlated with fasting blood glucose level (r = 0.95, P = 0.000; Fig. 6E) and infarct volume (r = 0.87, P = 0.006; Fig. 6F) but inversely correlated with cMVD in the nonischemic cortex (r = -0.90, P = 0.000; Fig. 6G) and the in peri-infarct area (r = -0.86, P = 0.008; Fig. 6H). Similarly to EPC-MPs, the level of circulating EMPs was positively associated with fasting blood glucose (r = 0.95, P = 0.000; Fig. 6I) and cMVD (0.87, P = 0.006; Fig. 6J) but negatively correlated with cMVD in the nonischemic cortex (r = -0.88, P = 0.003; Fig. 6K) and the in peri-infarct area (r = -0.88, P = 0.003; Fig. 6L).

Circulating db/db MPs but not db/+ MPs impair the functions of EPCs. EPCs were cultured from the BM of db/+ mice (wild-type controls). EPCs were preincubated with vehicle,
db/+ MPs, or db/db MPs for 1, 12, or 48 h before migration and tube formation assays (n = 4/group). The data of EPC migration and tube formation in the vehicle groups of each time point were not significantly changed and were defined as 100% (migration: 100 ± 2, 100 ± 2, and 100 ± 4%; tube formation: 100 ± 2, 100 ± 3, and 100 ± 3% at 1, 12, and 48 h, respectively; Fig. 7, A and B). For comparisons, the data in different preincubation groups at each time point were expressed as percentage of the corresponding vehicle group. As shown in Fig. 7, A and B, the EPC functions were not significantly changed after 1 h of preincubation with db/db MPs (migration ability: 98 ± 2%; tube formation ability: 95 ± 4%; vs. vehicle, P > 0.05) or with db/+ MPs (migration ability: 99 ± 2%; tube formation: 94 ± 5% vs. vehicle, P > 0.05). EPC functions remained unchanged after preincubation with db/+ MPs for 12 (migration ability: 95 ± 4%; tube formation ability: 95 ± 4%; vs. vehicle, P > 0.05; vs. 1 h, P > 0.05) and 48 h (migration ability: 92 ± 6%; tube formation ability: 91 ± 6%; vs. vehicle, P > 0.05; vs. 1 or 12 h, P > 0.05). However, EPC functions were much impaired after preincubation with db/db MPs for 12 (migration ability: 55 ± 6%; tube formation ability: 60 ± 6%; vs. vehicle or db/+ MPs, P < 0.01; vs. 1 h, P < 0.01) and 48 h (migration ability: 25 ± 3%; tube formation ability: 24 ± 4%; vs. vehicle or db/+ MPs, P < 0.01; vs. 1 or 12 h, P < 0.01).

Infusion of EPCs preincubated with db/db MPs has less efficacy in increasing the level of cEPCs and fails to reduce cerebral ischemic damage. The effects of EPC infusion on the levels of cEPCs and cerebral ischemic injury were determined using EPCs preincubated with db/+ MPs, or db/db MPs for 48 h (n = 6/group). Data showed that infusion of EPCs preincubated with db/+ MPs significantly increased the levels of cEPCs (1.6 ± 0.2 and 3.0 ± 0.04 numbers/μl; vs. vehicle, P < 0.01; Fig. 7C) and reduced the infarct volume (31.2 ± 2.5 and 38.5 ± 3.8%, vs. vehicle, P < 0.05; Fig. 7D), whereas infusion of the EPCs preincubated with db/db MPs had less efficacy in increasing the levels of cEPCs (0.9 ± 0.06 numbers/μl; vs. vehicle or infusion of EPCs preincubated with db/+ MPs, P < 0.05; Fig. 7C) and failed to reduce the infarct volume (36.8 ± 3.1%; vs. vehicle) or infusion of EPCs preincubated with db/+ MPs, P > 0.05; Fig. 7D).

**DISCUSSION**

There are several novel findings in the present study. First, db/db type 2 diabetic mice have higher levels of circulating EPC-MPs, EMPs, and decreased cMVDs. Second, db/db mice have less elevation of cEPCs after ischemic stroke. Third, the level of cEPCs is correlated negatively with fasting blood glucose and infarct volume but positively with cMVDs. The levels of circulating EPC-MPs and EMPs have opposite correlations with these indices. Fourth, BM-derived EPCs are reduced in number and are dysfunctional in db/db mice. Fifth, circulating MPs from db/db diabetic mice impair EPC functions, and infusion of EPCs preincubated with db/db MPs fails to reduce ischemic damage in db/db mice.

Although it is well known that the outcome of ischemic stroke is worse in patients with diabetes mellitus, the underlying mechanisms have not been well understood (25, 50). There is also little information on ischemic stroke in diabetic animal models. In this work, we used db/db mice as a type 2 diabetic animal model for an in vivo ischemic stroke study. As expected, we found that cerebral ischemic damage is exaggerated in this mouse model. We further observed that cMVDs in both the nonischemic cortex and the peri-infarct area of the brain are reduced in db/db mice. It has been suggested that the peri-infarct region is an important target for reducing ischemic damage following cerebral ischemia (17) and that impairment
in microcirculation contributes to the development of cerebral ischemic damage (28). These data can account for the exaggerated ischemic damage seen in \( db/db \) mice and may also reflect the impaired cerebral angiogenesis in diabetes. Asahara and colleagues (5, 6) first isolated EPCs and evaluated their contribution to angiogenesis after tissue ischemia. It is well accepted to define EPCs as CD34\(^+\)/H11001VEGFR\(^+\)/H11001 cells using the flow cytometric method (23, 55). To explore the possible implication of cEPCs in diabetes, we used the flow cytometric method to quantify the number of cEPCs and found that the level of cEPCs is decreased in \( db/db \) diabetic mice. In addition, we found that EPCs derived from \( db/db \) mice BM are reduced in generation and dysfunctional with regard to migration and tube formation. These findings are in agreement with previous reports in diabetic patients (13, 21, 43, 52) as well as recent studies in streptozotocin-induced type 1 (27) and \( db/db \) type 2 diabetic mice (44). Previous observations indicate that high glucose affects several aspects of EPCs; for example, it reduces EPC generation and proliferation, enhances EPC senescence, and impairs EPC migration and tube formation functions (13, 36). However, we should note here that we cultured EPCs in a medium containing normal instead of high glucose concentration. Our EPC culture results strongly suggest that the reductions in cEPCs and cMVD seen in \( db/db \) mice could result from reduced EPC generation and impaired function of cEPCs in diabetes.

EPCs are mobilized from BM into the peripheral circulation upon stimulation by cellular factors (3) and ischemia (27, 34). As expected, we found that the levels of cEPCs in both \( db/db \) and \( db/+ \) mice are increased 48 h after ischemic stroke. Of interest, the levels of cEPCs are less increased in \( db/db \) mice. One possible explanation for this result is that EPC generation/mobilization from BM in response to ischemia is reduced in \( db/db \) mice (27, 56). This is supported by our in vitro EPC generation culture study showing that BM-derived EPCs are reduced and dysfunctional in \( db/db \) diabetic mice. Several signal pathways have been shown to regulate EPC mobilization from BM. The downstream signals of hypoxia inducible factor-1\( \alpha \) (HIF-1\( \alpha \)), SDF-1\( \alpha \), and VEGF are considered to be the major factors for mobilizing EPCs under ischemia. Upregulation of the HIF-1\( \alpha \)/SDF-1\( \alpha \) pathway in the ischemic tissue is important for successful EPC mobilization, homing, and tissue repair (16, 51). In addition, endothelial nitric oxide synthase-derived nitric oxide (NO) in BM ECs and osteoblasts participates in regulating EPC mobilization (2). In diabetes, defective EPC mobilization following peripheral tissue ischemia or injury has been shown to associate with the inability to upregulate HIF-1\( \alpha \) downstream factors such as SDF-1\( \alpha \) (10, 41), VEGF, and NO (27). Our previous study indicates that SDF-1\( \alpha \) expression in the brain of \( db/db \) mouse is decreased in basal and in response to MCAO-induced ischemic stroke (6a). This evidence helps us to interpret the current data. Another possible explanation is that risk factors present in diabetes may increase EPC senescence (31) and deteriorate EPC function.

**Fig. 6.** Levels of cEPCs, EPC-MPs, and EMPs are correlated with fasting blood glucose, cerebral microvascular density (MVD), and infarct volume. *Left:* the level of cEPCs is negatively correlated with fasting blood glucose level (A) and infarct volume (B) but is positively correlated with cerebral MVD in nonischemic cortex (C) and in peri-infarct area (D). *Middle:* the level of cEPC-MPs is positively correlated with the fasting blood glucose (E) and infarct volume (F) and is negatively correlated with cerebral MVD in nonischemic cortex (G) and in peri-infarct area (H). *Right:* the level of circulating EMPs (cEMPs) is positively correlated with fasting blood glucose (I) and infarct volume (J) and is negatively correlated with cerebral MVD in nonischemic cortex (K) and in peri-infarct area (L).
CIRCULATING EPCs AND CELL MEMBRANE MPS IN DIABETIC STROKE

Fig. 7. Preincubation of EPCs with db/db MPs impairs the EPC functions, and infusion of db/db-incubated EPCs is less effective in increasing the level of cEPCs and fails to reduce cerebral ischemic damage in db/db mice. A and B: preincubation of EPCs with db/db MPs impairs EPC migration (A: *P < 0.01 vs. vehicle or db/+ MPs) and tube formation (B: *P < 0.01 vs. vehicle or db/+ MPs) in a time-dependent manner (#P < 0.01 vs. 1 h or 12 h; n = 4/group), whereas the functions of EPCs preincubated with db/+ MPs are not significantly changed. C: infusion of db/db MP-incubated EPCs is less effective in increasing the level of cEPC-preincubated EPCs (*P < 0.05; **P < 0.01 vs. vehicle; #P < 0.05 vs. infusion of EPCs preincubated with db/+ MPs). D: infusion of db/db MP-preincubated EPCs fails to reduce infarct volume (*P < 0.05 vs. vehicle; #P < 0.05 vs. infusion of EPCs preincubated with db/db MPs; n = 6/group). Data are expressed as means ± SE.

A

B

C

D

(23, 27). Nonetheless, we did not determine the lifespan and the level of apoptosis of EPCs. The latter possibility deserves future investigation.

It is accepted that cEPCs play an important role in angiogenesis and vascular homeostasis. BM-derived EPCs play an important role in maintaining vascular integrity (53). Numerous studies demonstrate correlations between the levels of cEPCs and vascular disease risks (31, 54). Decline in cEPC number and function has been shown to be associated with reduced reendothelialization ability after vascular injury in diabetes (43, 52). In the present study, a positive association of cEPCs with cMVD and a negative association of cEPCs with fasting blood glucose are demonstrated. Our findings further verify previous clinical observations showing that the number of cEPCs negatively correlates with the severity of diabetes (21, 23, 54) and that glucose tolerance is negatively associated with the level of circulating progenitor cells (22). In addition, our data showed that the level of cEPCs negatively correlates with ischemic infarct volume. This result may help to explain a previous report showing that the level of cEPCs is associated with outcome of ischemic stroke (49). From the current results, we tentatively assume that the lower level of cEPCs leads to reduced reendothelialization ability after vascular injury in db/db diabetic mice.

The EPCs release EPC-MPs under various situations such as stress and apoptosis (18, 48). In the present study, we found the elevated level of circulating EPC-MPs in db/db mice. This observation supports the concept that elevated levels of circulating MPs can be used as biomarkers for various cardiovascular diseases (37, 42). We also observed a further increase in circulating EPC-MPs following ischemic stroke, which may be a result of increased numbers of cEPCs. The higher level of circulating EPC-MPs and the lower level of EPCs in db/db mice may indicate a greater degree of EPC fragmentation into MPs. Pirro et al. (48) recently showed that the level of circulating EPC-MPs is positively correlated with aortic stiffness in patients. In this study, positive correlations of circulating EPC-MPs with fasting blood glucose and infarct volume, as well as a negative correlation with cMVD, are detected in the db/db mice. We provide evidence for supporting the potential of EPC-MPs for predicting ischemic stroke in diabetes.

Cardiovascular risk factors contribute to EC injury and induce EMP production (14, 33). In our study, the level of circulating EMPs is increased in db/db diabetic mice, suggesting the presence of vascular endothelial damage. We did not observe any significant alternation in the level of circulating EMPs following ischemic stroke, unlike in previous reports, which show increased circulating EMPs following ischemic stroke and cardiovascular events (33, 37). This data is not hard to be interpreted because of the fact that we induced an acute ischemic stroke in db/db mice by MCAO surgery, which should not have any effect on endothelial damage on top of diabetes. In contrast, accelerated EC damage usually happens in patients with spontaneous ischemic stroke. Similar to circulating EPC-MPs, we found positive correlations of circulating EMPs with fasting blood glucose and infarct volume and a negative correlation with cMVD in db/db mice. Our results imply that circulating levels of EMPs could be another predictive biomarker of ischemia stroke in diabetes.

To further determine the possible implication of cEPCs and circulating MPs, we conducted in vitro and in vivo experiments to test our hypothesis that levels of cEPCs and MPs may play a causal role in ischemic damage in db/db mice. First, we studied the role of circulating MPs in EPC functions. We found that preincubation of circulating MPs from db/db diabetic mice with EPCs time-dependently impairs EPC migration and tube formation (Avila et al. 2015). This result may help to explain the lower level of cEPCs in db/db diabetic mice. First, we studied the role of circulating MPs in EPC functions. We found that preincubation of circulating MPs from db/db diabetic mice with EPCs time-dependently impairs EPC migration and tube formation.
formation. Conversely, MPs from db/+ mice have no such effect on EPC functions. The data indicate that MPs from diabetic mice are detrimental to EPC function. With regard to the notorious effect of MPs, it is consistent with a previous report that shows that circulating MPs from metabolic syndrome patients induce endothelial dysfunction (1). This result also adds new evidence for supporting the concept that MPs carry the characters from their parent cells and mediate a beneficial or detrimental role by communication/interaction with other cells (45). Second, we further tested the in vitro findings by determining the efficacies of in vivo infusion of EPCs preincubated with MPs on ischemic damage in db/db mice. We found that transfusion of db/+ MPs incubated EPCs (with normal function, as shown in the in vitro data) and can increase the level of cEPCs and reduce ischemic damage in db/db mice. The data are in agreement with two previous reports. Kalka et al. (38) reported that transplant of human EPCs has the effects in improving blood flow recovery, capillary density, and the outcome in a rodent model with hindlimb ischemia. Most recently, Fan et al. (24) showed that EPC transplantation reduces mouse cortex atrophy after MCAO and improves neurobehavioral outcomes potently via increasing angiogenesis in the per-infarction area. Interestingly, we found that transfusion of EPCs preincubated with db/db MPs has fewer efficacies in increasing the level of cEPCs and reducing infarct volume compared with those infused with EPCs preincubated with db/+ MPs. Taken all together, the current data of our functional and therapeutic experiments suggest a casual involvement of circulating MPs on EPC functions and implicate that circulating EPC reduction and/or dysfunctions could be involved in the exaggerated cerebral ischemic damage seen in db/db mice. However, the detailed mechanisms were not fully explored in this study.

In summary, results of this study demonstrate that the brains of db/db animals have reduced cMVD, which may account for the increased ischemic damage seen in these diabetic mice. The reduced number of cEPCs and the impaired function of EPCs might be responsible for the decreased cMVD in the reduced number of cEPCs and the impaired function of EPCs explored in this study.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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