Transfusion of CXCR4-Primed Endothelial Progenitor Cells Reduces Cerebral Ischemic Damage and Promotes Repair in db/db Diabetic Mice

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Transfusion of CXCR4-Primed Endothelial Progenitor Cells Reduces Ischemic Damage and Promotes Repair in db/db Diabetic Mice

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Abstract

This study investigated the role of stromal cell-derived factor-1α (SDF-1α)/CXC chemokine receptor 4 (CXCR4) axis and explored the efficacy of CXCR4 primed EPCs in treating ischemic stroke in diabetes. The db/db diabetic and db/+ plasma SDF-1α and circulating CD34+CXCR4+ cells were measured. Brain SDF-1α and CXCR4 expression were quantified in db/db mice undergoing MCAO. In an in vitro study, EPCs were transfected with adenovirus carrying null (Ad-null) or CXCR4 (Ad-CXCR4) for 4 days. For pathway block experiments, cells were pre-incubated with PI3K inhibitor or nitric oxide synthase inhibitor and CXCR4 expression, function and apoptosis were determined. The p-Akt/Akt and p-eNOS/eNOS expression in EPCs transfected with Ad-null or Ad-CXCR4 were infused into mice via tail vein. On day 2 and 7, the cerebral blood flow, neovascular density, angiogenesis and neurogenesis were determined. We found: 1) The levels of plasma SDF-1α decreased in db/db mice; 2) The basal level of SDF-1α and MCAO-induced up-regulation of SDF-1α/CXCR4 axis were increased by Ad-CXCR4 transfection; 3) Ad-CXCR4 transfection increased CXCR4 expression in EPCs and enhanced EPC colony forming capacity; 4) Ad-CXCR4 HG-induced dysfunction (migration and tube formation) and apoptosis via activation of PI3K/Akt/eNOS signal pathway. EPC efficacy of EPC infusion in attenuating infarct volume and promoting angiogenesis and neurogenesis. Our data suggest therapeutic effects for ischemia stroke in diabetes than unmodified EPCs do.

Figures

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Introduction

Diabetes is a risk factor for stroke, which are the nation's second leading cause of death and the leading cause of long cerebral damage is exacerbated and the outcome is poor. The responsible mechanisms might include microvascular impaired angiogenesis. Endothelial progenitor cells (EPCs) are believed to play an important role in maintaining endothelial participate in angiogenesis which represents an important endogenous tissue repair mechanism [1], [2]. Accumulating reduced in number and impaired in function in diabetic patients and animals [3]–[5]. Studies on ischemic brain, heart able to reduce tissue injury, promotes angiogenic repair and functional recovery [3], [6], [7]. These positive results pro ischemic stroke in diabetes.

The stromal cell-derived factor-1α (SDF-1α)/CXCR chemokine receptor 4 (CXCR4) axis is believed to play an important tissue [8]–[10] and triggers many intracellular proliferation and anti-apoptosis signals, such as mitogen-activated prote 3-kinase (PI3K) and the serine/threonine kinase Akt [11]. Therefore, it is a potential target for promoting repair in W ischemic heart and limbs have shown that a combination of SDF-1α/CXCR4 over-expression and stem cell transduction ischemic diseases. SDF-1α pretreatment increases the therapeutic potential of EPC transduction in a mouse model of ischemic stroke in diabetes. CXCR4 in mesenchymal stem cells enhances in vivo engraftment into the ischemic heart and subsequently improves f myoangiogenesis [13]. When compared to low-CXCR4-expressing EPCs, administration of high-CXCR4-expressing E promotes blood flow recovery in ischemic hindlimbs [14]. However, there is little information on EPCs-based therapy f ischemic diseases. SDF-1α/CXCR4 signal pathway is dysregulated in the brain of db/db diabetic role of CXCR4/PI3K/Akt/eNOS signaling pathway and high glucose (HG) in EPC function and survival. Furthermore, w Ad-CXCR4 primed EPCs is more effective on treating ischemic stroke in db/db mice.

Material and Methods

Animal Experimental Design

Adult male db/db diabetic mice (C57BL6/J) and their age matched (8–10 weeks) controls (db/+) were used for the st Maine). The general characteristics of db/+ and db/db mice are summarized in Table 1. The db/db mice possess an ir receptor and subsequently develop obesity, hyperglycemia and insulin resistance resembling adult-onset diabetes mel used mouse model for type 2 diabetes [15]. The level of fasting plasma glucose was measured after 16 hours fasting Monitor (Roche Diagnostic, Indianapolis, IN). All experimental protocols (Figure 1) were approved by the Laboratory / Wright State University and Guangdong Medical College in accordance to the Guide for the Care and Use of Laboratc Health.
Figure 1. Experimental protocols.
The flow diagrams briefly describe the in vitro and in vivo protocols.
doi:10.1371/journal.pone.0050105.g001

Table 1. General Characteristics of db/+ and db/db Mice.
doi:10.1371/journal.pone.0050105.t001

Protocol one.

For exploring whether SDF-1α/CXCR4 axis is dysregulated in the brain of diabetes (at basal and after ischemic stroke) randomly assigned to middle cerebral artery occlusion (MCAO) or sham surgery group. MCAO surgery was performed we previously described [3], [16]. Mice were euthanized 48 hours after surgery, and the brain tissues were immediate contralateral side hemispheres were dissected for analysis of SDF-1α and CXCR4 expression. For real-time RT-PCR into 1 ml tubes containing 0.5 ml RNAlater (Qiagen, CA) and cut into small pieces (<0.5 cm³). After overnight, tissues were immediately harvested into tubes and put on dry ice before they were transferred.

Protocol two.

For determining the therapeutic efficacy of Ad-CXCR4 primed EPCs on ischemic stroke in diabetes, bone marrow (BM) EPCs were cultured for 7 days and then transfected with adenovirus (Ad) carrying null (Ad-null-EPCs) or CXCR4 gene transfection, EPCs were continuously cultured for another 2 days to expansion. After that, the cells were harvested, cultured subjected to MCAO surgery (under anesthesia by inhaling 2.5% isoflurane) and randomly assigned to different treatment solutions, PBS), Ad-null-EPCs and Ad-CXCR4-EPCs. Pain and discomfort were minimized by an initial injection of Buprenorphine, another two injections every 12 hours. Mice were injected via the tail vein with EPCs (2×10^6 cells/100 µl in PBS) or the controls. To label the new generated cells, mice were injected with bromodeoxyuridine (BrdU, 65 µg/g/day, i.p.) immediately before experimental endpoint [18]. Neurologic motor function was determined and cerebral blood flow (CBF) was measured right before mice (n = 12/group/time point) were euthanized on day 2 or 7. Blood samples were taken from the heart under deep anesthesia (pentobarbital, 150 mg/kg body weight) [3]. For real-time RT-PCR analysis of CXCR4 expression (n = 6/group; ischemic hemisphere were immediately harvested as described in Protocol one. For histological analysis (n = 6/group; 4% paraformaldehyde (PFA). Then, brain tissues were fixed in 4% PFA plus 30% sucrose for 3 days. Fixed brains were (20 µm) and divided into four wells for Fluoro-Jade staining analysis of infarct volume, and immunohistological analysis angiogenesis and neurogenesis.
Enzyme-linked Immunosorbent Assay (ELISA) for SDF-1α

The plasma level of SDF-1α was measured by ELISA methods [19]. Briefly, mouse plasma was collected and detected using the high capacity cDNA archive kit (Qiagen). The real-time PCR was run with the following primer sequences: CXCR4 (5′-TTT CAG CCA GCA GTT TCC TT-3′ and 5′-TCA GTG GCT GAC CTC CTC TT-3′); St GTG GTG-3′ and 5′-AGA GCT GGG CTC CTA CTG TGC GGC CGC GGG-3′). β-actin was chosen for housekeeping expression.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The levels of SDF-1α and CXCR4 of the brain tissues were determined using real-time RT-PCR methods [20]. Brain tissue was isolated from the db/+ and db/db mice were count coated 24-well plates (BD Bioscience, San Jose, CA, USA) and then grown in endothelial cell basal medium-2 (EBM-2) growth cytokine cocktail (Lonza, Walkersville, MD, USA). After 3 days in culture, non-adherent cells were removed by washing with PBS and the adherent cells were transfected with Ad-null or Ad-CXCR4 (1×10^7 infectious units) viruses were removed and the medium was replaced with fresh medium with FCS in the following day. Cells were confluency for harvest. CXCR4 expression in EPCs was confirmed by real-time RT-PCR and western blot. The percentage of CXCR4+ EPCs was determined by flow cytometry (Accuri C6 flow cytometer, Inc. Ann Arbor, MI) after staining EPCs with anti-CXCR4 (CXCR4-PE, eBioscience). Colony forming units (CFUs) were counted by visual inspection with an inverted microscope, defined as a central core of round cells with elongated sprouting cells at the periphery, as previously reported [22].

High Glucose Experiments on EPCs

The HG (25 mmol/L) medium which corresponds to 350–450 mg/dl of plasma glucose levels in diabetic patients was used for osmotic control as previous reports [24]–[25]. Ad-null-EPCs or Ad-CXCR4-EPCs were cultured in high glucose supplemented with SDF-1α (100 ng/ml) for 4 days before functional assays. The medium were changed every two da level of the culture supernatant was daily monitored by an oxidase-based colorimetric method [27] during the HG exposure pre-incubated with PI3K inhibitor (LY294002, 20 µM, Cell Signaling) or NOS inhibitor (Nω-nitro-arginine methyl ester, LMA) for two hours [28].
EPC Migration and Tube Formation Assays

EPC migration and tube formation were evaluated by using Boyden chamber (Chemicon, Rosemont, IL) and tube formation previously described [3]. For migration, EPCs (2×10^4 cells) were placed into upper compartment of the Boyden chamber and 100 ng/ml stromal cell-derived factor-1 (SDF-1) in the lower compartment. EPCs were allowed to migrate across the membrane were counted under an inverted light microscope, quantified and averaged by examination (magnification, ×200). For tube formation, ECMatrix solution was thawed on ice overnight, mixed with 10×ECMatrix dilution, and placed on 37°C for one hour to allow the matrix solution to solidify. EPCs were re-plated (1×10^4 cells/well) on top of the matrix and allowed to migrate for 48 hours at 37°C. Tube formation was evaluated with an inverted light microscope and defined as a tube structure exhibiting independent fields were assessed for each well, and the average number of tubes per field (magnification, ×200) was determined. EPCs were cultured in the basal EPC medium as previously described [30].

EPC Apoptosis Assay

After 4 days' culture in HG medium, EPCs were harvested for apoptosis analysis by using Alexa Fluor 488 annexin V/propidium iodide (Invitrogen, Carlsbad, CA). Briefly, cells were re-suspended in annexin-binding buffer, and then incubated with annexin V/propidium iodide at room temperature (RT). The apoptotic EPCs were recognized as PI−/Annexin V+ cells. The percentage of apoptosis was a

Western Blot Analysis

Gene expression of SDF-1α, CXCR4, eNOS, Akt, p-eNOS or p-Akt of the brain tissue or EPCs was determined [28], using antibodies against SDF-1α (1:200, Roche Diagnostic) containing protease inhibitor. The proteins were subjected to SDS-PAGE electrophoresis and transferred to nitrocellulose membranes were blocked by incubating with 5% dry milk and Tris-buffered saline for one hour, and then incubated with primary antibodies against CXCR4 (1:100, AnaSpec Inc. CA), Akt (1:1000, Cell Signaling Technology), eNOS (1:1000, Cell Signaling Technology), or p-eNOS (1:1000) at 4°C overnight. β-actin (1:4000, Sigma, MO) was used to normalize protein loadings were incubated with horseradish peroxidase (HRP) conjugated IgG (1:40000, Jackson Lab) for one hour at RT. Blots were chemiluminescence developing solutions and quantified.

Flow Cytometry Analysis of Circulating EPCs and CD34+CXCR4+ Cells

The level of circulating EPCs was determined by flow cytometry as a previous study [3]. Briefly, circulating MNCs were harvested and stained with anti-mouse CD34-PE (AbD Serotec, Raleigh, NC) and VEGFR2-PE-Cy7 (BD, Bioscience) antibodies. CD34+CXCR4+ cells, circulating MNCs were stained with CD34-FITC (AbD Serotec) and CXCR4-PE (eBioscience, San Diego, CA), and stained with anti-mouse CD34-PE (AbD Serotec, Raleigh, NC) and VEGFR2-PE-Cy7 (BD, Bioscience) antibodies. CD34+CXCR4+ cells, circulating MNCs were stained with CD34-FITC (AbD Serotec) and CXCR4-PE (eBioscience, San Diego, CA). CD34+CXCR4+ cells were expressed as cells/ml blood. Isotype (IgG) antibodies were used as a

Functional Evaluation of Neurological Deficits

The neurological deficit scores were evaluated on day 2 or 7 after EPC treatment for functional determination of therapy. The neurological scale method was previously described [16], [32]. The neurological scale for mice was scored by an investigator who was unaware of animal grouping.

Measurement of Cerebral Blood Flow

On day 2 or 7 following EPC transfusion, the relative CBF in the peri-infarct area was determined as described previously. Mice were anesthetized with 2.5% isoflurane and placed on a stereotaxic apparatus. An incision was made in the scalp at the peri-infarct site of ischemic ipsilateral area (2 mm posterior, 6 mm lateral to bregma) and contralateral site (2 mm posterior, 6 mm lateral to bregma) as a point of reference for calibration. To minimize variability, the CBF was recorded at each site for at least 5 minutes. The averaged volume CBF for each site. The relative CBF was calculated using the formula: relative CBF = CBF of ipsilateral side/CBF of contralateral side.
performed CBF measurements was unaware of the information of animal grouping.

Measurement of Infarct Volume and Cerebral Microvascular Density

As we previously described [3], [16], cerebral ischemic damage and the cMVD in peri-infarct area were revealed by staining with Fluoro-Jade (0.001%, Histo-chem, Jefferson, AR, USA) and CD31 (1:50, Invitrogen), respectively. Infarct volume and software (NIH).

Analysis of Angiogenesis and Neurongenesis

Angiogenesis and neurongenesis in peri-infarct area were determined by using double immunofluorescence staining with antibodies: CD31 (1:50, Abcam, MA, USA), followed by incubation with cell-specific antibodies: CD31 (1:50, BD Biosciences), GFAP (1 overnight at 4°C). Next, brain sections were reacted with FITC (for BrdU) or Cy3 (for cell specific markers) conjugated 30 min at RT in the dark. The labeled ECs (BrdU+CD31+), neurons (BrdU+NeuN+) and glial cells (BrdU+GFAP+) in the peri-infarct area were counted under 6 random fields (200×). The average of five sections from rostral to caudal represented the data for each group, counted by an investigator who was unaware of animal grouping.

Statistical Analysis

All data, excepting neurologic deficit scores, are presented as mean ± SE. The neurologic deficit scores were expressed as a percentage of the maximum score among different groups were compared by the Kruskal–Wallis test. When the Kruskal–Wallis test showed a significant difference, comparisons were applied. For the rest measurements, comparisons for two groups were performed by the student’s t test. Multiplicity was controlled by two-way ANOVA. For all tests, a P-value <0.05 was considered significant.

Results

Baseline Characterization of Animals

The characterizations of blood glucose, age and body weight in db/db and db/+ mice used in this study are presented in Table 2. db/db mice had higher plasma glucose and body weight as compared with age-matched db/+ control mice. In protocol, surgery (blood flow <75% of baseline) and randomized to vehicle, Ad-null-EPC or Ad-CXCR4-EPC infusion groups. Table 2 shows blood glucose among different treatment groups (Table 2).

| Groups               | B.W. (g) | Blood glucose (mg/dl) |
|----------------------|----------|-----------------------|
| Vehicle, 2 day       | 48.2±2.1 | 428.5±10.4            |
| Vehicle, 7 day       | 47.9±2.2 | 425.8±11.2            |
| Ad-null-EPCs, 2 day  | 43.6±1.5 | 421.6±12.4            |
| Ad-null-EPCs, 7 day  | 42.2±1.5 | 418.4±11.8            |
| Ad-CXCR4-EPCs, 2 day | 46.9±1.6 | 424.8±12.2            |
| Ad-CXCR4-EPCs, 7 day | 46.6±1.1 | 421.5±12.4            |

Data are means ± SE, n = 6/group. B.W.: Body weight.
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Table 2. Baseline Characteristics of db/db Mice in Different Groups.
The Levels of Plasma SDF-1α and Circulating CD34+CXCR4+ Cells are Reduced in db/db Mice

The level of plasma SDF-1α was significantly lower in db/db mice (1.3±0.14 and 1.8±0.15 pg/ml, P<0.05, db/db vs. db/+ mice). The number of CD34+CXCR4+ cells was reduced in db/db mice (260±14 and 712±42 cells/ml, P<0.01, db/db vs. db/+ mice, n = 9/group).

The Expression of SDF-1α/CXCR4 Axis is Dysregulated in the Brain of db/db Mice at Stroke

At basal, the db/db diabetic mice had less expression of SDF-1α in the brain tissue at both mRNA and protein levels (P<0.05; Figure 2B and D). The levels of brain SDF-1α and CXCR4 in the ischemic ipsilateral hemisphere were up-regulated in both db/db and db/+ mice 48 hours following MCAO (P<0.05 or 0.01). However, the up-regulations of SDF-1α/CXCR4 were not observed in the contralateral hemisphere (P>0.05; Figure 2). The levels of brain SDF-1α and CXCR4 in the contralateral hemisphere was unaffected (data not shown).

Figure 2. SDF-1α/CXCR4 expression in the brain of db/db mice at basal and in response to ischemia. (A) SDF-1α mRNA expression. (B) CXCR4 mRNA expression. (C) SDF-1α protein expression. (D) CXCR4 protein expression. *P<0.05, **P<0.01 vs. sham; # P<0.05 vs. db/+, n = 5/group in mRNA analysis, n = 3/group in protein analysis. doi:10.1371/journal.pone.0050105.g002

EPC Characterization and CXCR4 Expression in EPCs

BM derived EPCs were defined as cells up-taking Di-LDL and binding with Bs-Lectin, as well as cells expressing CD34 (Figure 3A and B). At the end of EPC culture (7 days), the percentage of CD34+VEGFR2+ EPCs was 89±3.5% (n = 5/group). However, the percentage of CXCR4+ EPCs did not change significantly over the 7-day culture period (P>0.05). The CXCR4+ EPCs were enriched in the conditioned medium of stimulated acellular matrix (AAM) compared to the control medium (P<0.05; Figure 3C).

Figure 3. Characterization of bone marrow derived EPCs.
Ad-CXCR4 Transfection Increases CXCR4 Expression and Colony Forming Capacity

Real-time PCR and western blot analyses showed that Ad-CXCR4 transfection up-regulated CXCR4 expression in EPC protein levels (P<0.01; Figure 4A and B). Flow cytometric result showed that Ad-CXCR4 transfection significantly increased CXCR4 expression (P<0.01; Figure 4C). The number of CFUs was decreased in EPCs from db/db mice (P<0.05 or 0.01; Figure 4D). Ad-CXCR4 transfection from both db/+ and db/db mice (P<0.01; Figure 4D).

Ad-CXCR4 Transfection Protects EPCs from HG-induced Dysfunction and Apoptosis

PI3K/Akt/eNOS Signaling Pathway

HG incubation for 4 days significantly decreased the expression of CXCR4 in EPCs from both db/+ and db/db mice (P<0.05; Figure 4E). HG incubation also induced down-regulation of p-Akt and p-eNOS and increased the expression of Akt and eNOS in EPCs (Figure 6). Pre-incubation of Ad-CXCR4 transfection on EPC function and apoptosis (P<0.05 or 0.01), Whereas, NOS inhibitor (L-NAME) (P<0.01; Figure 5).
Figure 5. Ad-CXCR4 transfection protects down-regulation of Akt/eNOS activation in EPCs induced by HG

(A) Representative western blot bands showing Akt/eNOS and p-Akt/p-eNOS expression in different treatment groups. Akt and p-Akt, and 140 kDa for eNOS and p-eNOS. (B) Summarized data on Akt/eNOS and p-Akt/p-eNOS expression. *P<0.05, **P<0.01 vs. Ad-null-EPCs; ***P<0.01 vs. Con or Osm. Con: control (basal medium); Osm: osmotic control. PI3K: phosphatidylinositol-3-kinase; NOS: nitric oxide synthase; Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.
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Figure 6. Ad-CXCR4 transfection protects EPCs from HG-induced dysfunction and apoptosis via activation

Representative tube formation pictures (A1–A8) and summarized data (A9) in different treatment groups. A1: Ad-null-EPCs+HG; A2: Ad-null-EPCs+Con; A3: Ad-null-EPCs+Osm; A4: Ad-CXCR4-EPCs+Con; A5: Ad-CXCR4-EPCs+Osm; A6: Ad-CXCR4-EPCs+HG; A7: Ad-CXCR4-EPCs+HG+L-NAME. Scale bar: 600 µm. Summarized data on migration ability (B) and the percentage of Ad-null-EPCs and Ad-null-EPCs+Osm; **P<0.01 vs. HG+Ad-null-EPCs; #P<0.05, ##P<0.01 vs. HG+Ad-CXCR4-EPCs+LY294002, n = 6/group. Con: control (basal medium); Osm: osmotic control; HG: high glucose. Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.
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Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing the Level of Expression in the Brain

The db/db mice were treated with EPCs two hours after MCAO surgery. Infusion of Ad-null-EPCs was able to increase CXCR4 expression in the brain of ischemic side on day 7 (P<0.05) with no significant change in infusion of Ad-CXCR4 primed EPCs was more effective to increase CXCR4 expression in the ischemic hemisphere or
Figure 7. Effects of Ad-CXCR4-EPC infusion on cEPCs, brain CXCR4 expression and cMVD in db/db mice. (A) The level of cEPCs in each therapeutic group. (B) The CXCR4 expression in the brain of db/db mice in each therapeutic group. (C) Pictures of cMVD (CD31 immunostaining) in the peri-infarct area. Scale bar: 50 µm. (D) The level of cMVD in the therapeutic group. *P<0.05, **P<0.01 vs. vehicle; *P<0.05, **P<0.01 vs. Ad-null-EPCs; $P<0.05$ vs. day 2, n = 6/group.

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing cMVD in the Damage

Infusion of Ad-null-EPCs was able to increase the cMVD in peri-infarct area in db/db mice (Day 2, $P<0.05$; Day 7, $P<0.01$) after transfusion of Ad-CXCR4 primed EPCs could enhance the efficacy ($P<0.01$; Figure 7C and D).

Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Increasing Relative CBF Injury and Neurologic Deficit Score

In agreement with the findings in cMVD, we also found that Ad-null-EPC transfusion improved the relative CBF of peri-infarct area (Figure 8A) and transfusion of Ad-CXCR4 primed EPCs was more effective (Day 2, $P<0.05$; Day 7, $P<0.01$; Figure 8A) after Ad-null-EPC infusion, and was able to be further decreased after the both day 2 and day 7 ($P<0.01$; Figure 8B). To evaluate the neurologic motor function, we measured neurologic deficit score. We found that the neurologic deficit score was reduced in Ad-null-EPC group on day 7 ($P<0.01$; Figure 8C).
Figure 8. Effects of Ad-CXCR4-EPC infusion on CBF, infarct volume and neurologic deficit score in db/db mice.

(A) The relative CBF in peri-infarct area in each therapeutic group. (B) The infarct volume in each therapeutic group. *P<0.05, **P<0.01 vs. vehicle; **P<0.05, ***P<0.01 vs. Ad-null-EPCs; $P<0.05$ vs. day 2, n = 6/6/6/group. Ad-null-EPCs: EPCs transfected with Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.

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Infusion of Ad-CXCR4 Primed EPCs Enhances the Efficacy in Promoting Angiogenesis

Figure 9A shows representative pictures of angiogenesis (BrdU+CD31+), glial (BrdU+GFAP+) and neuronal (BrdU+NeuN+) genesis in db/db mice following infusion of Ad-CXCR4-EPCs. Data showed that Ad-null-EPC transfusion increased angiogenesis and neurogenesis on day 7 (P<0.05 or 0.01; Figure 9B–E). Moreover, transfusion of Ad-CXCR4 primed EPCs promoted angiogenesis as early as day 2 (P<0.01), and had better neurogenesis on day 7 (P<0.01 or 0.05; Figure 9B–D).

Figure 9. Infusion of Ad-CXCR4-EPCs increases angiogenesis and neurogenesis in db/db mice following islet transplantation. (A) Representative pictures of angiogenesis (BrdU+CD31+), neurogenesis (BrdU+NeuN+) and glial cell genesis (GFAP) after Ad-CXCR4-EPC treatment. Scale bar: 50 µm. Histogram showing the number of BrdU+CD31+ (B), BrdU+NeuN+ (C), and GFAP (D) cells in peri-infarct area on day 2 and 7 in different therapeutic groups. *P<0.05, **P<0.01 vs. vehicle; **P<0.05, ***P<0.01 vs. Ad-null-EPCs. NeuN: neuronal nuclei; GFAP: glial fibrillary acidic protein; Ad-null-EPCs: EPCs transfected with Ad-null; Ad-CXCR4-EPCs: EPCs transfected with Ad-CXCR4.

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Discussion

There are three major findings in this present study. Firstly, we found that the expression of SDF-1α/CXCR4 axis is dy
at basal and in response to ischemic stroke. Secondly, we illustrated that Ad-CXCR4 primed EPCs display resistance through activation of CXCR4 downstream PI3K/Akt/eNOS signal pathway. Thirdly, we demonstrated that infusion of A efficacy in reducing ischemic injury as well as promoting recovery.

The SDF-1α regulates tissue/organ homeostasis through the CXCR4 receptor expressed in hematopoietic progenitors SDF-1α expression is reduced at basal and that ischemia-induced up-regulation of brain SDF-1α and CXCR4 are less first evidence showing the dysregulation of SDF-1α/CXCR4 axis in the brain of an animal model with diabetes. A previous SDF-1α and CXCR4 is up-regulated in the tunica media of the thoracic aortas in streptozotocin-induced type-1 diabetic diabetic models have hyperglycemia, we tentatively attribute this inconsistency to different animal models and/or tissue are supported by other previous reports showing that the SDF-1α/CXCR4 axis is down-regulated in the wounds of db/db model used in our study has hyperglycemia, obesity and dyslipidemia and insulin resistance [15]. Therefore, it deserve besides hyperglycemia can also lead to the impairment of SDF-1α/CXCR4. Our in vitro data showed that HG down-regulated the SDF-1α/CXCR4 axis is impaired at multiple sites (brain and EPCs) in diabetes, which might have repair (enlarged injury and delayed repair); targeting on the dysfunction of SDF-1α/CXCR4 axis could offer a new avenue.

In addition, we found that the levels of plasma SDF-1α and circulating CD34+CXCR4+ cells are reduced in db/db mice diabetic patients [40]. Since the SDF-1α/CXCR4 interaction triggers several intracellular signals including MAPKs, PI3 modulate cell migration, proliferation and apoptosis [11], we investigated the implication of SDF-1α/CXCR4 axis in EP expected, we found that Ad-CXCR4 transfection protects EPCs from HG-induced dysfunction and apoptosis. The und CXCR4 downstream PI3K/Akt/eNOS signal pathway since PI3K or eNOS inhibitor abolishes or partially blocks these interaction with previous observations showing that SDF-1α/CXCR4 interaction mediates EPC migration via Akt and e data suggest that the SDF-1α/CXCR4 axis is impaired at multiple sites (brain and EPCs) in diabetes, which might have repairing function, and induced EPC apoptosis. Although diabetes is a stage of severe inflammation and oxidative stress, and the mimic the situation in in vivo, our results are supported by the reports from others [30], [23] showing that HG induced data are also in agreement with our previous findings showing lower level of cEPCs and less cerebral microvascular dysfunction hyperglycemia should be one of the mechanisms for EPC dysfunction in diabetes. On the other hand, evidence suggest vascular progenitors from the bone marrow [38], [39]. The db/db mice possess an inactivating gene mutation in leptin current findings in db/db mice.

EPCs have been found to differentiate into ECs and contribute to angiogenic repair [42], [43]. A recent report demons infarct volume in ischemic stroke mice [7]. Yang et al [44] also demonstrated that CD34+ cells could represent a funct beneficial therapeutic effects in myocardial infarction. In patients with diabetes and db/db diabetic mice, circulating EP dysfunctional [3]–[5]. Our previous study demonstrates that transfusion of EPCs from non-diabetic sources has benefi reports by others also suggest that EPCs be useful for therapeutic purposes in diabetes [21], [45]. Because of the metabolic factors in diabetic patients [40]. Since the SDF-1α/CXCR4 interaction triggers several intracellular signals including MAPKs, PI3 modulate cell migration, proliferation and apoptosis [11], we investigated the implication of SDF-1α/CXCR4 axis in EP expected, we found that Ad-CXCR4 transfection protects EPCs from HG-induced dysfunction and apoptosis. The und CXCR4 downstream PI3K/Akt/eNOS signal pathway since PI3K or eNOS inhibitor abolishes or partially blocks these interaction with previous observations showing that SDF-1α/CXCR4 interaction mediates EPC migration via Akt and e data suggest that the SDF-1α/CXCR4 axis is impaired at multiple sites (brain and EPCs) in diabetes, which might have repair (enlarged injury and delayed repair); targeting on the dysfunction of SDF-1α/CXCR4 axis could offer a new avenue.

The EPCs are thought to be a mixture of progenitor cells and mononuclear cells. At present, isolation of pure population characterization of the different types of EPC is currently an open issue with debate [48]. However, the generally acce expression of surface markers including CD34, CD133 and KDR [49]. In this study, we cultured EPCs for 7 days and found the percentage of CD34+VEGFR2+ cells was about 88.5%, suggesting most of them are EPCs. Moreover, also important to obtain the high purity EPCs. We isolated BM MNCs by gradient density separation method. BM MNC 24-well plates and grown in endothelial cell basal medium-2 containing EPC growth cytokine cocktails in favor of the p
lines [21], [50]. After 3 days of culture, non-adherent cells were removed by washing with PBS to avoid contamination. The observed effects are attributed to the transfused EPCs, rather than the CD45+ mononuclear cells.

Our in vitro EPC culture and in vivo animal studies are in a good agreement for supporting the beneficial effects of Ac stroke. Firstly, Ad-CXCR4 transfection protects EPCs from HG induced apoptosis resulting in increased level of circulating EPCs prevents EPCs from HG-induced dysfunction (migration and tube formation) and leads to the promotion of angiogenesis increases angiogenesis in peri-infarct area as early as day 2, whereas transfection of EPCs shows this effect on day 7 of CMVD and relative CBF on day 2 in EPC treatment group. Although the underline mechanism is unclear, we tentatively which secrete angiogenic factors promoting the proliferation and survival of resident ECs. Another major finding of our more effective than non primed EPCs in promoting cerebral repair processes. This is evidenced by increased angiogenesis in the Ad-CXCR4 primed EPC treatment group.

In summary, the present study demonstrates that transfusion of Ad-CXCR4 primed EPCs may be a novel approach for stroke in diabetes. Over-expression of CXCR4 in EPCs prevents the deleterious effects of HG on EPC function and angiogenesis which could be the underlying mechanism for the beneficial effects of Ad-CXCR4 primed EPC transfusion. Here, we determine the level of EPC incorporation into endothelium, the level of local SDF-1α after EPC transfusion, and the parameters deserve future investigation.

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**Author Contributions**

Conceived and designed the experiments: Ji Chen Jianying Chen BZ YC. Performed the experiments: Ji Chen Jianying Chen. Analyzed the data: Ji Chen Jianying Chen SC CZ XX LZ AD YZ. Contributed reagents/materials/analysis tools: MM. Wrote the paper: Ji Chen MM YC.

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