Impaired therapeutic vasculogenesis by transplantation of OxLDL-treated endothelial progenitor cells

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Abstract Previous in vitro studies have revealed that oxidized low density lipoprotein (OxLDL) has negative effects on the proliferation and activity of endothelial progenitor cells (EPCs). Here, we evaluated the effect of OxLDL on the therapeutic potential of EPCs in ischemia-induced neovascularization. EPCs derived from mobilized human peripheral blood mononuclear cells were cultured without or with OxLDL before transplantation. Hindlimb ischemia models were surgically induced in athymic nude mice, which then received an intracardiac injection of 3×10^5 EPCs. By laser Doppler perfusion image and ischemia damage score, we found that blood perfusion and ischemia damage were less well recovered in the OxLDL-treated EPC transplantation group than in controls. Histological examination showed fewer transplanted EPCs and lower capillary density in ischemic tissue. Local delivery of Stromal cell-derived factor (SDF-1) restored this defect and improved blood perfusion by recruiting OxLDL-treated EPCs to the ischemic area and increasing host capillary density. These results provide for the first time direct evidence that OxLDL impaired the therapeutic potential of EPCs in ischemia-induced neovascularization through an inhibitory effect on the migration, adhesion, and incorporation of EPCs into vasculature and/or entrapment in the perivascular region in vivo. A therapeutic strategy based on SDF-1 administration ameliorated such defects and improved postischemic neovascularization.—Zhou, B., F. X. Ma, P. X. Liu, Z. H. Fang, S. L. Wang, Z. B. Han, M-C. Poon, and Z. C. Han. **Impaired therapeutic vasculogenesis by transplantation of OxLDL-treated endothelial progenitor cells. *J. Lipid Res.* 2007.** 48: **518–527.**

Supplementary key words oxidized low density lipoprotein • ischemia • stromal cell-derived factor

The ubiquitous blood vessel system is vulnerable to many pathological processes, such as atherosclerosis and diabetes mellitus (1). In the late stage of these diseases, patients usually suffer from severe limb ischemia. Thus, prevention and treatment of tissue ischemia is important. The classical mechanism of postnatal neovascularization had been considered to be limited to angiogenesis until endothelial progenitor cells (EPCs) were discovered (2). The finding that EPCs are home to sites of neovascularization and differentiate into endothelial cells (ECs) in situ is consistent with "vasculogenesis," a critical paradigm well described for embryonic neovascularization but recently proposed in adults (3). Our previous work indicated that transplantation of ex vivo expanded EPCs from either peripheral or cord blood could enhance neovascularization in hindlimb ischemia of nude mice (4, 5). However, the number of circulating EPCs and their migratory activity were reported to be reduced in patients with risk factors for ischemic cardiovascular disease or to be negatively correlated with the Framingham cardiovascular risk factor score (6, 7). Circulating EPCs serve as a biological marker for vascular function (7), and reduction of the number of circulating EPCs predicts future cardiovascular events (8). Given the concept of therapeutic vasculogenesis contributed by EPCs, further understanding of the regulation of EPC kinetics may bring new insights into the pathogenesis of vasculogenesis.

Oxidized low density lipoprotein (OxLDL) is one of factors that influence the growth and bioactivity of EPCs. Previous work indicated that OxLDL has negative effects on the number and activity of EPCs in vitro, inhibiting EPC differentiation and inducing EPC senescence, leading to cellular dysfunction (9–11). Recently, we showed that OxLDL inhibited EPC survival and impaired its function, and this action was attributable to an inhibitory effect on endothelial nitric oxide synthase (12). However, the exact therapeutic potential of these OxLDL-treated EPCs (OxLDL-EPCs) in ischemic settings is little known. We hypothesize that OxLDL not only directly injures ECs but also impairs the repair process by EPCs (therapeutic vasculogenesis) in vivo. Therefore, we studied the ability of OxLDL-EPCs in treating severe hindlimb ischemia to

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demonstrate the impact of OxLDL on EPCs both in vivo and in vivo.

METHODS

Cell preparation

Mobilized peripheral blood mononuclear cells (M-PBMNCs) were collected from healthy volunteers who received 600 μg/day recombinant human granulocyte colony-stimulating factor (Kirin Pharmaceuticals, Tokyo, Japan) subcutaneously for 5 days to mobilize stem/progenitor cells. All volunteers had no risk factors of coronary artery diseases, including hypertension, diabetes, smoking, positive family history of coronary artery disease, and hypercholesterolemia, and were free of wounds, ulcers, retinopathy, recent surgery, inflammation, malignant diseases, or medications that may influence EPC kinetics. Human umbilical cord was manipulated according to the standard protocol approved by the Institutional Review Board of the Institute of Hematology and the Hospital of Blood Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College. All involved volunteers were well informed and provided signed informed consent.

Preparation and oxidation of LDL

Blood samples for LDL preparation were taken from healthy volunteers after 12 h of fasting. Written informed consent was obtained from all volunteers in accord with the Institutional Ethics Committee. LDL was separated by density gradient ultracentrifugation as described previously (12). After isolation by density gradient ultracentrifugation, LDL was oxidized by exposure to 5 μmol/l $CuSO_4$ for 18 h at 37°C. LDL was totally oxidized, because there is only one band by electrophoresis on agarose gels. Compared with native LDL, the OxLDL showed increased electrophoretic mobility and an increased level of thiobarbituric acid-reactive substances (TBARS). The level of TBARS was 2.23 ± 1.56 and 24.37 ± 8.14 nmol/mg protein in native LDL and OxLDL, respectively. Compared with the native LDL, OxLDL showed increased electrophoretic mobility on agarose gels of 1.8 ± 0.4 times. OxLDL was sterilized by passing it through a 0.22 mm filter.

Cell isolation and culture

Mobilized peripheral blood mononuclear cells were isolated from blood of human volunteers by density gradient centrifugation with Histopaque-1077 (Sigma). Cells were plated on culture dishes coated with human fibronectin (Sigma) and maintained in EC Basal Medium-2 (EBM-2) (Clonetics, Cambrex), supplemented with EGM-2 MV SingleQuots containing 5% FBS, human vascular endothelial growth factor (VEGF), human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and hydrocortisone. After 4 days in culture, nonadherent cells were removed and new medium was added. The culture was maintained through day 7. After 7 days of culture, EPCs were stimulated with or without OxLDL (50 μg/ml) for 24 h. Then, OxLDL-EPCs were used in the following experiments in comparison with normal EPCs. Human umbilical vein endothelial cells (HUVECs) were isolated by incubating umbilical vein with 0.25% trypsin for 15 min at 37°C and were cultured in M199 with 20% FBS and 1% L-glutamine. HUVECs in passages 3–5 were used. To examine the effect of SDF-1 on OxLDL-EPC survival, we supplemented the culture with 100 ng/ml SDF-1 for 12 h, with medium as a control.

EPC characterization

After 7 days in culture, adherent cells ($2–3 \times 10^5$) were immunostained with anti-human antibodies: CD31, vWF, VE-cadherin, KDR, CD34, CD45, CD3, and CD19. Phycoerythrin-conjugated monoclonal antibodies against CXCR4 were used to detect CXCR4 expression on EPCs. Isotype-matched mouse immunoglobulin served as a control. Cells were quantitatively analyzed using FACSCalibur™ and CellQuest software (BD Biosciences). For the purposes of this study, attached cells that showed uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-AcLDL) and binding of FITC-*Ulex europaeus* agglutinin (UEA) were considered EPCs. Briefly, cells were incubated with 10 μg/ml Dil-AcLDL (Molecular Probes) at 37°C for 4 h and fixed with 2% paraformaldehyde for 10 min. After washing, cells were then incubated with 10 μg/ml FITC-UEA-1 (Sigma) at 4°C for 30 min. Adherent cells were dyed for the nuclei with 4′,6-diamino-phenylindole (DAPI) and washed twice. The incorporation of Dil-AcLDL and binding of FITC-UEA-1 were detected by confocal microscopy (Leica Microsystems GmbH).

EPC transplantation animal model

All protocols were performed on male athymic nude mice (7–8 weeks old, 15–19 g; Institute of Experimental Animals, Beijing, China) according to Peking Union Medical College Animal Use and Care guidelines. Mice were anesthetized with 60 mg/kg sodium pentobarbital intraperitoneally before surgery. Left femoral artery, great saphenous artery, iliac circumflex artery/vein, and its muscular branch were ligated to induce left hindlimb ischemia (13). One day after the establishment of hindlimb ischemia, mice received an intracardiac injection of 3×10^5 culture-expanded EPCs (normal) or OxLDL-EPCs (n = 15 in each group). To track the fate of the administered cells, five mice in each group received cells labeled with CM-Dil (Molecular Probes). Before transplantation, adherent cells were washed and incubated with 2 μg/ml CM-Dil for 5 min at 37°C and 15 min on ice. After washing with PBS twice, cells were resuspended in EBM-2 and injected into mice (100 μl/mouse). At 30 min before euthanasia, these mice received an intracardiac injection of either 50 μg of *Bandeiraea simplicifolia* lectin I (BS lectin-1; Vector Laboratories) or UEA-1 (Vector Laboratories). To assess the therapeutic potential of SDF-1, 15 athymic nude mice in each group received a local intramuscular injection of 1 μg of SDF-1 versus PBS in the center of the lower calf muscle followed immediately by an intravenous injection of 3×10^5 OxLDL-EPCs or normal EPCs as a control.

Physiological assessment of transplanted animals

Laser Doppler perfusion imaging (LDPI; Lisca AB, Linkoping, Sweden) was used to assess the extent of blood flow restoration in mice after surgery, as described previously (4). Measurements were done before surgery and at days 1, 3, 7, 14, and 28 after operation. The images were subjected to computer-assisted quantification of blood flow, and the perfusion index was expressed as the ratio of left (ischemic) to right (nonischemic) limb blood flow. A semiquantitative functional assessment of the ischemic limb was performed in a blinded manner using a modification of a clinical score (1) (0 = toe flexion, 1 = foot flexion, 2 = no dragging but no plantar flexion, 3 = foot dragging). Ischemic damage was also scored (0 = no change, 1 = mild discoloration, 2 = moderate/severe discoloration, 3 = necrosis, 4 = amputation) (14), and all scoring was done by a blinded observer.

Histological assessment of transplanted animals

Tissue sections from the lower calf muscles of ischemic and healthy limbs were harvested on days 3, 7, and 28 and snap-frozen

in liquid nitrogen. Frozen sections of 5 mm thickness were mounted on silane-coated glass slides and air-dried for 1 h. To examine EPC incorporation at early time points after transplantation (at days 3, 7, and 14), tissues from the mice injected with Dil-labeled EPCs and BS lectin-1 were embedded for frozen section samples. A total of 20 different fields (4 fields in each section, 5 cross-sections from each animal) were randomly selected, and the Dil-labeled EPCs were counted. Tissues from ischemic and normal hindlimb muscles on days 3 and 7 were also examined for the expression of adhesive molecules. Sections were probed with goat anti-mouse P-selectin and E-selectin (ZhongShan Biotech, Beijing, China) and incubated with PE-conjugated rabbit anti-goat secondary antibody (ZhongShan Biotech).

Migration assay

The chemotactic effect of VEGF and SDF-1 on OxLDL-EPCs was assessed by the in vitro chemotaxis assay in a Transwell chamber (8 μm pores; Coastar, Cambridge, MA). VEGF or SDF-1 diluted to 1, 10, or 50 ng/ml in 600 μl of EBM-2 containing 0.5% BSA was placed in the lower compartment, and 10^5 EPCs were suspended in the upper compartment. After incubation for 3.5 h at 37°C, the filters were removed, and the cells in the lower compartment were obtained and counted using flow cytometry with appropriate gating for 20 s at a high flow rate. The migratory rate was determined by calculating the percentage of input cells migrating into the lower chamber (average events for 20 s/average input cell events for 20 s × 100%). All groups were studied in triplicate.

Apoptosis assay

EPC apoptosis, induced by serum starvation, was assessed by annexin V/propidium iodide binding assay as described previously (12). To exclude dead cells, only annexin V-positive and propidium iodide-negative cells were counted. We also used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) to detect in situ apoptosis according to the manufacture's protocol (Roche Molecular Biochemicals). Nuclei were stained with DAPI, and the percentage of TUNEL-positive cells was evaluated in a blinded manner.

Adhesion assay

To test the adhesion ability of EPCs after OxLDL treatment, either matrix molecules or ECs were used in our study. EPCs were incubated with 10 μg/ml Dil-AcLDL at 37°C for 4 h and gently detached with 0.1% trypsin. Identical numbers of EPCs and OxLDL-EPCs in EBM-2 containing 0.5% BSA were replated onto fibronection-coated 24-well culture plates, incubated for 1 h at 37°C, and then washed three times to remove nonadherent cells. For assay of EPC adhesion to ECs, a monolayer of HUVECs was prepared 48 h before the assay by plating 2×10^5 cells in each well of six-well glass slides. HUVECs were pretreated for 12 h with 1 ng/ml tumor necrosis factor-α (BD Biosciences). A total of 5×10^4 EPCs labeled with CM-Dil were added to each well and incubated for 3 h at 37°C. Nonattached cells were gently removed with PBS. Adherent EPCs were fixed with 4% paraformaldehyde stained with DAPI and counted in five random fields. The adherent EPCs on HUVECs were counted by fluorescence microscopy, and five fields of each well were assayed by independent blinded investigators.

Matrigel tubule assay

To investigate the ability of EPCs to integrate into vascular structures, EPCs were cocultured with HUVECs on matrigel (BD Biosciences). Briefly, matrigel was thawed at 4°C overnight and placed on a 24-well culture plate at 37°C for 1 h to allow solidification. EPCs treated without or with OxLDL were harvested and labeled with CM-Dil. Dil-EPCs (2×10^4) were coplated with HUVECs (4×10^4) labeled with PKH2-GL (Sigma) and incubated in EGM-2 MV at 37°C for 24 h. Tubule formation was defined as a structure exhibiting a length four times its width. The proportion of EPCs in tubules was determined in 10 random fields.

Western blot

Muscle samples were harvested at 3 or 7 days after surgery for VEGF assays. Tissues were lysed to collect protein, and the concentration was determined with the BCA™ protein assay kit (Pierce). Proteins were separated with the use of SDS-PAGE gels (12%) and incubated with antibodies to VEGF (1:250; Santa Cruz Biotechnology), Hypoxia-inducible factor-1(HIF-1)α (1:500; Santa Cruz Biotechnology), or actin (1:400; ZhongShan Biotech). Relative quantification of proteins was determined with the use of BanScan software.

Statistical analysis

Results are expressed as means ± SEM. The statistical significance of differences between groups was analyzed by ANOVA followed by Fisher's *t*-test for comparison between any two groups, except for CXCR4 expression, which was analyzed by paired *t*-tests. Statistical significance was assumed at a value of $P < 0.05$.

RESULTS

Attenuated therapeutic efficacy of OxLDL-EPC transplantation

Total mobilized peripheral blood mononuclear cells isolated and cultured for 1 week resulted in a spindle-shaped, EC-like morphology. Fluorescence-activated cell-sorting analysis confirmed the endothelial phenotype of the EPCs used in this study (**Fig. 1A**). The expression profile of cultured EPCs included CD31 (82.3 ± 5.3%), vWF (61.4 ± 8.8%), KDR (67.2 ± 8.3%), VE-cadherin (79.3 ± 6.4%), and CD34 (37.3 ± 8.4) and excluded the possible contamination by hematopoietic lineage cells, such as CD45 and T- or B-lymphocytes. These differentiating cells were also shown to endocytose Dil-labeled AcLDL and to bind FITC-conjugated UEA-1, consistent with endothelial lineage cells (Fig. 1B). The impact of EPC administration on therapeutic neovascularization was investigated in a murine model of hindlimb ischemia (13). To analyze subcutaneous blood perfusion, LDPI analysis was performed (15). Serial examination of hindlimb perfusion by LDPI performed at days 3, 7, 14, 21, and 28 disclosed profound differences in limb perfusion within 28 days after the induction of limb ischemia. Representative images are shown in Fig. 1C. At 1 or 3 days postoperatively, limb perfusion was severely reduced in both groups (P = NS). Over the subsequent 28 days, however, substantial blood flow recovery were observed in mice receiving normal EPCs. Limb perfusion did recover in mice receiving OxLDL-EPCs and was much better than in the PBS group, but to a lesser extent than that of the normal EPC group (Fig. 1D). By day 28, the ratio of ischemic to normal blood flow in OxLDL-EPC transplanted mice was 0.68 ± 0.37 vs. 0.82 ± 0.42 in mice receiving normal EPCs ($P < 0.05$). Limb

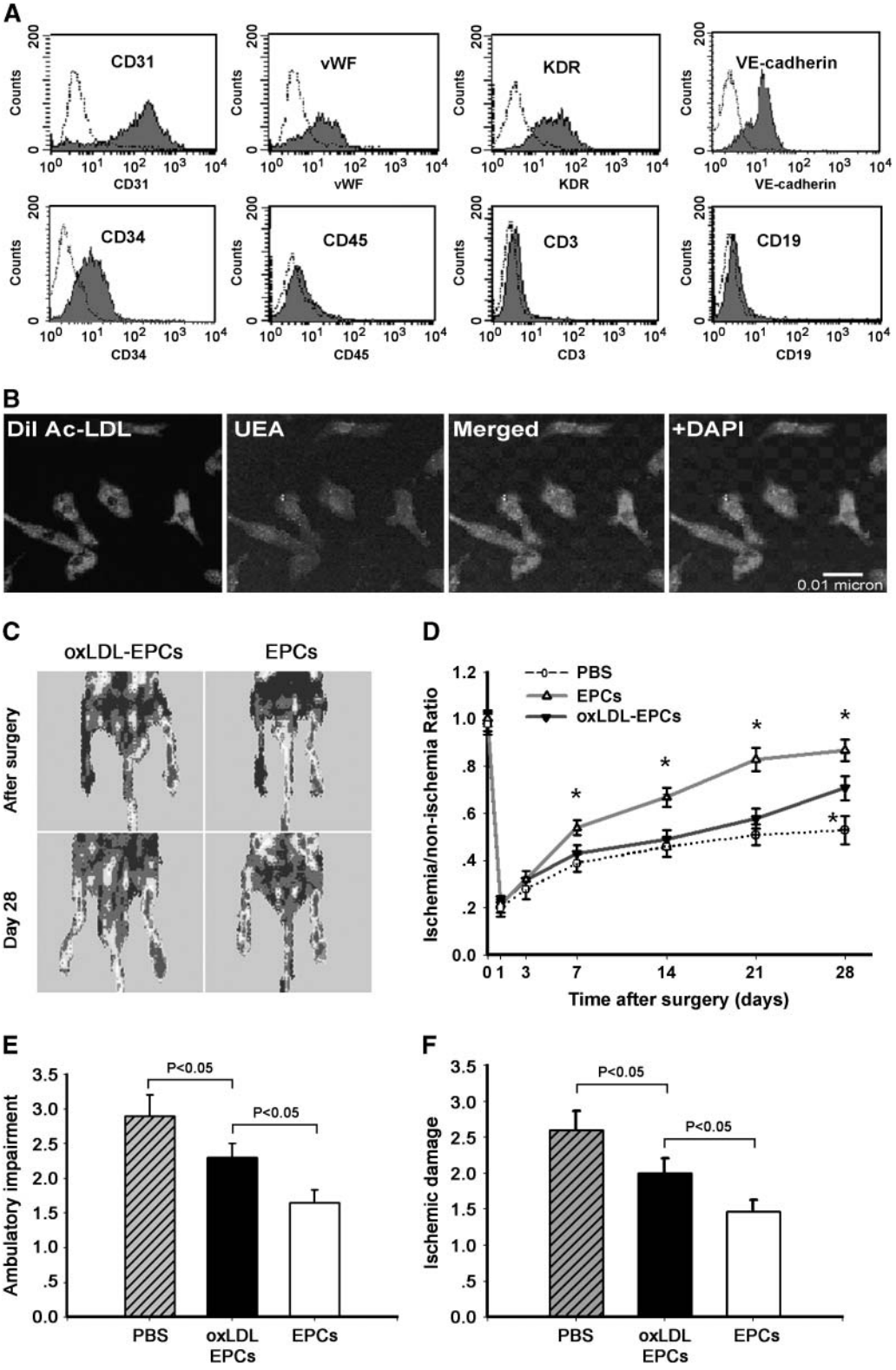

Fig. 1. Impaired therapeutic neovascularization after transplantation of oxidized low density lipoprotein (OxLDL)-treated endothelial progenitor cells (EPCs). A: Representative results of fluorescence-activated cell-sorting analysis of cell surface markers on EPCs collected from the adherent cell population. B: Expanded adherent cells [nucleus stained with 4′,6-diamino-phenylindole (DAPI)] could take up 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-AcLDL) and bind to FITC-*Ulex europaeus* agglutinin (UEA). Bar = 0.01 μm. C: Representative laser Doppler perfusion imaging (LDPI). In images, high blood flow is depicted in white, and low perfusion is dark. The left hindlimb was ischemic. D: Quantitative analysis of perfusion recovery as measured by the ischemia-nonischemia perfusion ratio at different time points (n = 15; * $P < 0.05$). E: Effects on ambulatory impairment based on ambulatory score (n = 15). F: Ischemic damage score of OxLDL-EPC- or normal EPC-treated groups (n = 15). For D–F, results are expressed as means ± SEM.

perfusion recovery influenced hindlimb appearance and function. Mice receiving OxLDL-EPCs, compared with those receiving normal EPCs, had significantly more impairment of function remaining at day 28 (OxLDL-EPCs vs. EPCs: ischemic score = 2.4 ± 0.19 vs. 1.7 ± 0.16; $P < 0.05$) (Fig. 1E) and more severe ischemic damage, with ischemia damage score 1.6-fold higher ($P < 0.05$) (Fig. 1F).

Homing and migration of OxLDL-EPCs into ischemic hindlimb neovasculature were impaired

It was reported that the participation and incorporation of transplanted EPCs into the capillary networks in the ischemic hindlimbs were responsible for the improved ischemia recovery (16). To elucidate the mechanisms of the impaired therapeutic potential of OxLDL-EPCs in vivo, we quantified the incorporation and/or entrapment of transplanted OxLDL-EPCs in the microvasculature or perivascular area of ischemic limbs and the number of host ECs. Dil-labeled OxLDL-EPCs were identified in tissue sections by red, whereas host ECs stained with BS lectin-1 were shown in green (**Fig. 2A**). Histological examination disclosed reduced local accumulation of OxLDL-EPCs compared with normal controls (day 3, 97 ± 9 vs. 126 ± 10 cells/mm^2; day 7, 118 ± 9 vs. 154 ± 9 cells/mm^2; day 14, 111 ± 8 vs. 154 ± 11 cells/mm^2; $P < 0.05$) (Fig. 2B). Likewise, a decreased number of mouse capillary ECs was observed in OxLDL-EPC-treated mice compared with normal EPC-treated mice (day 3, 321 ± 21 vs. 409 ± 25 cells/mm^2; day 7, 376 ± 23 vs. 487 ± 24 cells/mm^2; day 14, 478 ± 21 vs. 676 ± 27 cells/mm^2; $P < 0.05$) (Fig. 2C). Figure 2D represents the homing of Dil-labeled EPCs to ischemic tissues and differentiation into EC lineage cells (FITC-UEA) in vivo. Dil-labeled human EPCs (arrows) were identified around murine collateral artery (green) at 7 days after surgery (Fig. 2E). We also explored the possible mechanisms of homing of these EPCs to ischemic sites. By Western blot, we found that there were bright protein bands for mouse VEGF as well as HIF-1α in ischemic tissues at 3 or 7 days after surgery, but no measurable bands for normal hindlimb tissues (Fig. 2F). Upregulated VEGF, modulated by HIF-1α, could have chemoattractant potential to EPCs. When EPCs were subjected to chemotactic assay, they respond to stimulation of VEGF in a dose-dependent manner (Fig. 2G). However, the migratory activity of OxLDL-EPCs in response to VEGF was significantly reduced compared with that of normal EPCs (migration rate, 5.2 ± 0.34% vs. 6.8 ± 0.31%; $P < 0.05$) (Fig. 2H).

Attenuated adhesion and network formation ability of OxLDL-EPCs

We further investigated adhesion, which is an important step in the EPC homing process. Because EPCs were reported to express P-selectin glycoprotein ligand-1 PGSL-1, a ligand for P/E-selectin, adhesion molecules on ischemic ECs would have an important role in recruiting circulating EPCs to the murine vasculature. Immunohistochemistry revealed that ECs in the ischemic area expressed high levels of P-selectin and E-selectin (**Fig. 3A, B**). In vitro assessment of adhesion ability revealed that Dil-labeled OxLDL-EPCs were significantly impaired in their ability to adhere to fibronectin (Fig. 3C, D) or to a HUVEC monolayer activated with tumor necrosis factor-α (Fig. 3E, F). In vivo, EPCs stained with TRITC-UEA (red) were incorporated in the mouse vessel network stained with BS lectin-1 (green) in vivo (Fig. 3G, H). Matrigel tubule assay was performed to investigate the ability of EPCs to integrate into vascular structures. Dil-labeled OxLDL-EPCs (red) could actively incorporate into the PKH2-labeled HUVEC network structure (green) on matrigel (Fig. 3I, J). Dil-labeled OxLDL-EPCs (Fig. 3L) were found to contribute less to the tubule formation with HUVECs (transparent) compared with normal EPCs (Fig. 3K).

SDF-1 administration recruited more OxLDL-EPCs to ischemic limbs

When OxLDL-EPCs were subjected to chemotactic assay, they responded to stimulation with SDF-1 in a dose-dependent manner (**Fig. 4A**), although their migration was attenuated compared with that of normal EPCs. The impaired migration ability with SDF-1 was attributable to decreased CXCR4 expression in OxLDL-EPCs (data not shown). Because the impaired therapeutic vasculogenesis of OxLDL-EPC transplantation was related to the attenuated homing and migration to the ischemic area, we next determined whether SDF-1 administration in ischemic tissues could restore such defects and improve therapeutic vasculogenesis in mice. Compared with control mice systemically treated with OxLDL-EPCs and locally with PBS, mice systemically treated with OxLDL-EPCs and locally delivered with SDF-1 showed greater blood perfusion in ischemic hindlimbs at day 28 after surgery (n = 15; $P < 0.05$) (Fig. 4B, C), although less effective than the normal EPCs at days 21 and 28. To elucidate the SDF-1 effect on local recruitment of transplanted OxLDL-EPCs from the systemic circulation in vivo, we tested the incorporation of OxLDL-EPCs into murine microvasculature of ischemic limbs by histological examination. More Dil-labeled OxLDL-EPCs (red) and murine capillaries (green) were discovered in tissue sections after SDF-1 administration (Fig. 4D). There was a significantly increased accumulation of OxLDL-EPCs in SDF-1-treated tissues compared with the PBS group (day 3, 123 vs. 85 cells/mm^2; day 7, 158 vs. 112 cells/mm^2; day 14, 126 vs. 101 cells/mm^2; n = 5; $P < 0.05$) (Fig. 4E). Capillary density was also increased after administration of SDF-1 in ischemic tissue compared with PBS controls (day 3, 421 vs. 324 caps/mm^2; day 7,498 vs. 376 caps/mm^2; day 14,589 vs. 476 caps/mm^2; n = 5; $P < 0.05$) (Fig. 4F). In addition, SDF-1 level in plasma was increased in mice delivered locally with SDF-1 (Fig. 4G). Addition of SDF-1 in OxLDL-EPC culture significantly reduced apoptosis, compared with that of control medium ($P < 0.05$) (Fig. 4H).

DISCUSSION

Previous studies have demonstrated that transplantation of ex vivo expanded EPCs can improve blood flow recovery

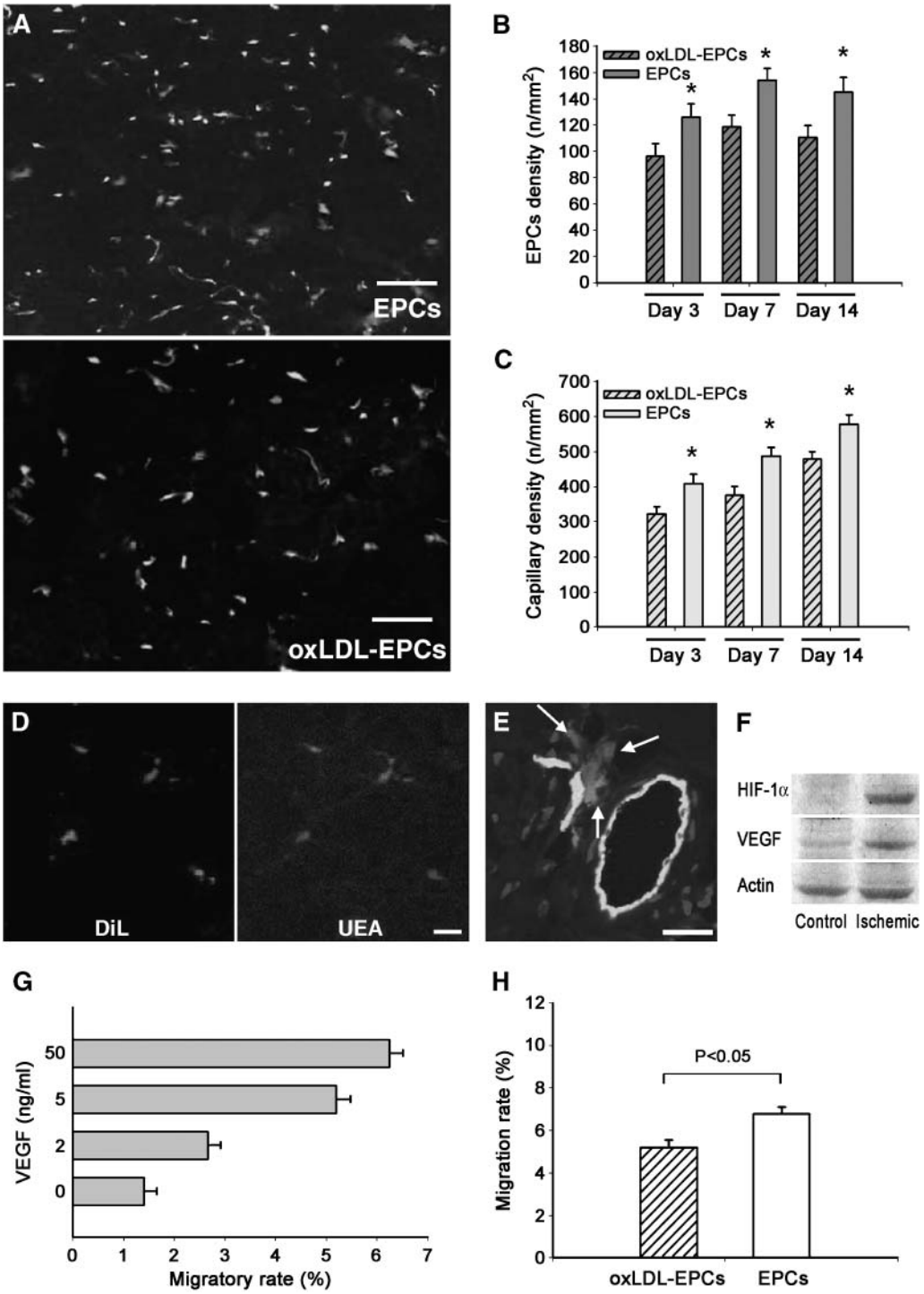

Fig. 2. EPC homing and migration to ischemic hindlimb vasculature. A: Representative micrographs of double fluorescence in ischemic muscles. Transplanted Dil-labeled EPCs were identified by (red fluorescence; grey) in histological sections retrieved from ischemic muscles. Host mouse vasculature was identified by (green fluorescence; white) in the same tissue sections. Bars = 50 μm. B: Quantitative analysis shows that the density of OxLDL-EPCs in ischemic tissue was less than that of normal EPCs at days 3, 7, and 14 (* $P < 0.05$). C: Quantitative analysis shows that the density of host endothelial cells in ischemic muscles was less in the OxLDL-EPC treatment group than in the normal EPC group at days 3, 7, and 14 (* $P < 0.05$). D: Human origin of the labeled cells was confirmed by the colocalization of CM-Dil and UEA, a human EC-specific binding lectin. Bar = 50 μm. E: Dil-labeled EPCs (grey) are found in the perivascular area [*Bandeiraea simplicifolia* lectin I (BS lectin-1)-labeled; white] in vivo. Nucleus is stained in blue. Bar = 50 μm. F: Strong HIF-1α and vascular endothelial growth factor (VEGF) protein induction in calf muscle of the hindlimb in which the femoral artery was ligated, and absence of HIF-1α and VEGF protein induction in nonischemic control limbs. G: The chemoattractant effect of VEGF on EPCs was dose-dependent. H: Fewer OxLDL-EPCs migrated toward the VEGF-containing lower chamber compared with normal EPCs. For B, C, G, and H, results are expressed as means ± SEM.

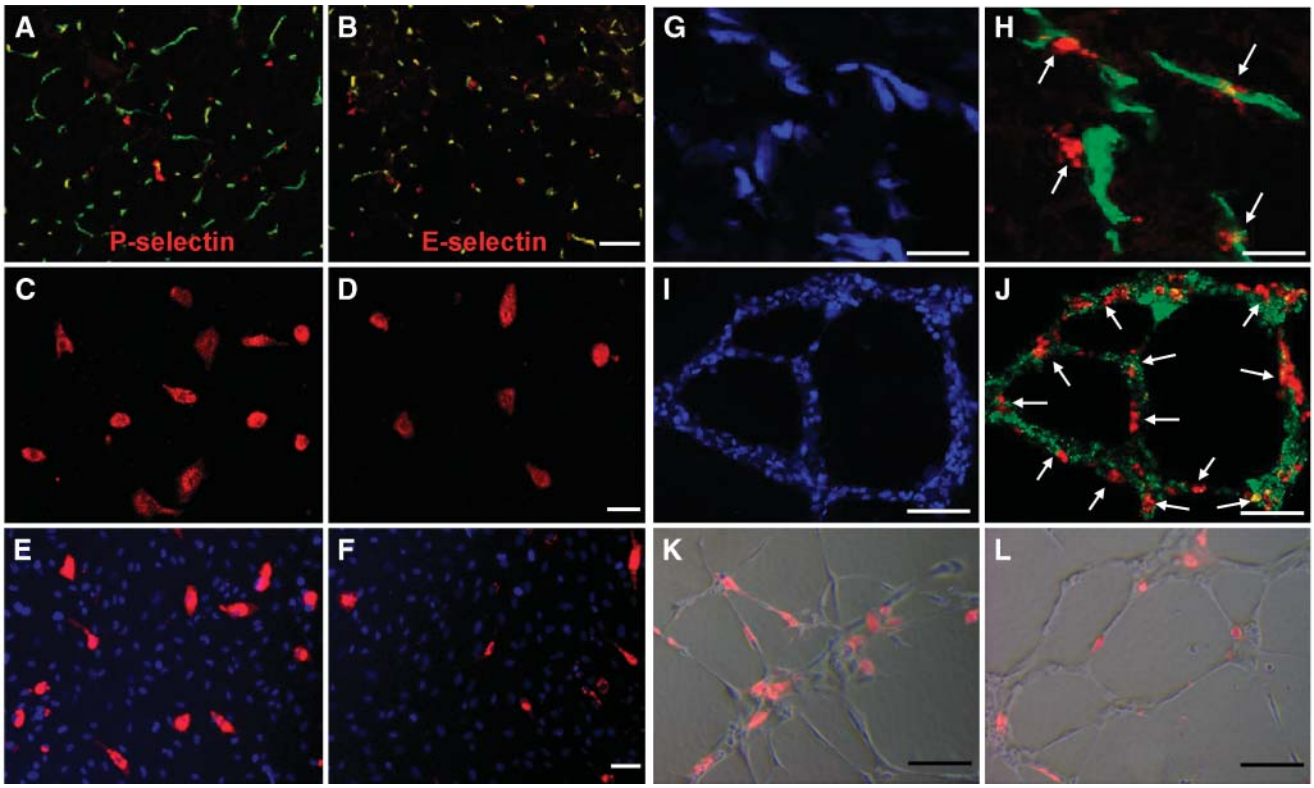

Fig. 3. EPC adhesion and vascular incorporation ability. A, B: With immunohistochemistry, P-selectin (A) and E-selectin (B) were expressed clearly on ischemic tissue. Bars = 100 μm (n = 5). C, D: Dil-labeled normal EPCs (C) and OxLDL-EPCs (D) were allowed to adhere to fibronectin-coated plates. Dil-labeled EPCs (red) were also allowed to adhere to a monolayer of human umbilical vein endothelial cells (HUVECs) for 3 h. Bars = 10 μm (n = 5). E, F: Representative images illustrating the adhesion of control EPCs (E) and OxLDL-EPCs (F) to tumor necrosis factor-α-activated HUVECs. The nucleus was stained with DAPI. Bars = 10 μm (n = 5). G, H: The incorporation of EPCs in vivo. In ischemic tissue, EPCs were stained red with TRITC-UEA, host ECs were stained green with FITC-BS lectin-1, and nucleus was stained blue with DAPI. Bars = 50 μm. I, J: In vitro, Dil-labeled EPCs (red; arrows) cocultured with PKH2-labeled HUVECs (green) actively participated in the tubule structures within matrigel. The nucleus was dyed with DAPI (blue). Bars = 50 μm (n =5). K: Superimposed light and fluorescent images revealed that control EPCs made a substantial contribution to tubule networks with HUVECs. Bars = 10 μm (n =5). L: In contrast, OxLDL-EPCs were less incorporated into the developing vascular network. Bar = 10 μm.

and capillary density in ischemic hindlimbs (4) and myocardial infarction (17). The transplanted EPCs can home to ischemic sites, differentiate, and incorporate into the vasculature (18). Thus, this beneficial property of EPCs is appealing for cell therapy targeting the regeneration of ischemic tissue. In this study, we found slow recovery of blood flow in ischemic hindlimbs after OxLDL-EPC transplantation, which subsequently resulted in severe ambulatory impairment and high ischemia damage. The fluorescence-labeled EPCs were found to home to the ischemic tissues and incorporate into murine vasculature, in accordance with previous results (19, 20). The number of recruited OxLDL-EPCs in ischemic sites in vivo as well as in host capillaries in ischemic tissue was much less than that of normal EPCs. This may be partly attributable to the reduced expression of CXCR4 in OxLDL-EPCs that could respond to increased SDF-1 in ischemic tissue. The impaired recruitment of OxLDL-EPCs might partly explain the slow recovery of blood flow and limb function. A recent study also indicated that recruitment and entrapment of circulating cells are vital steps in VEGF-induced angiogenesis (21). It is likely that EPCs were recruited to ischemic regions and participated in noeovascularization by the secretion of cytokines (18); thus, fewer EPCs residing or entrapped in ischemic sites may reduce the in vivo secretion of angiogenic factors that could augment therapeutic neovascularization. Whether OxLDL-EPCs are dysfunctional in secreting proangiogenic factors in vivo is unknown and merits further investigation in the future.

It has been demonstrated that there are multiple steps during the homing of transplanted hematopoietic stem/progenitor cells to bone marrow niches (5). The migration of target cells under specific chemoattractants and cell adhesion to local endothelium in conjunction with adhesive molecules are two initial and important steps (16). In our study, we found that ischemia induced an increase of HIF-1α, which upregulated the expression of VEGF. Besides this angiogenic role, VEGF has also been demonstrated to recruit bone marrow-derived angioblasts to sites of neovascularization (22). The recruited EPCs could also secrete angiogenic cytokines (18), including VEGF. Thus, release of VEGF by progenitor cells may result in a paracrine loop, supporting the proliferation of both endothelium and progenitors and recruiting more circulating EPCs. However,

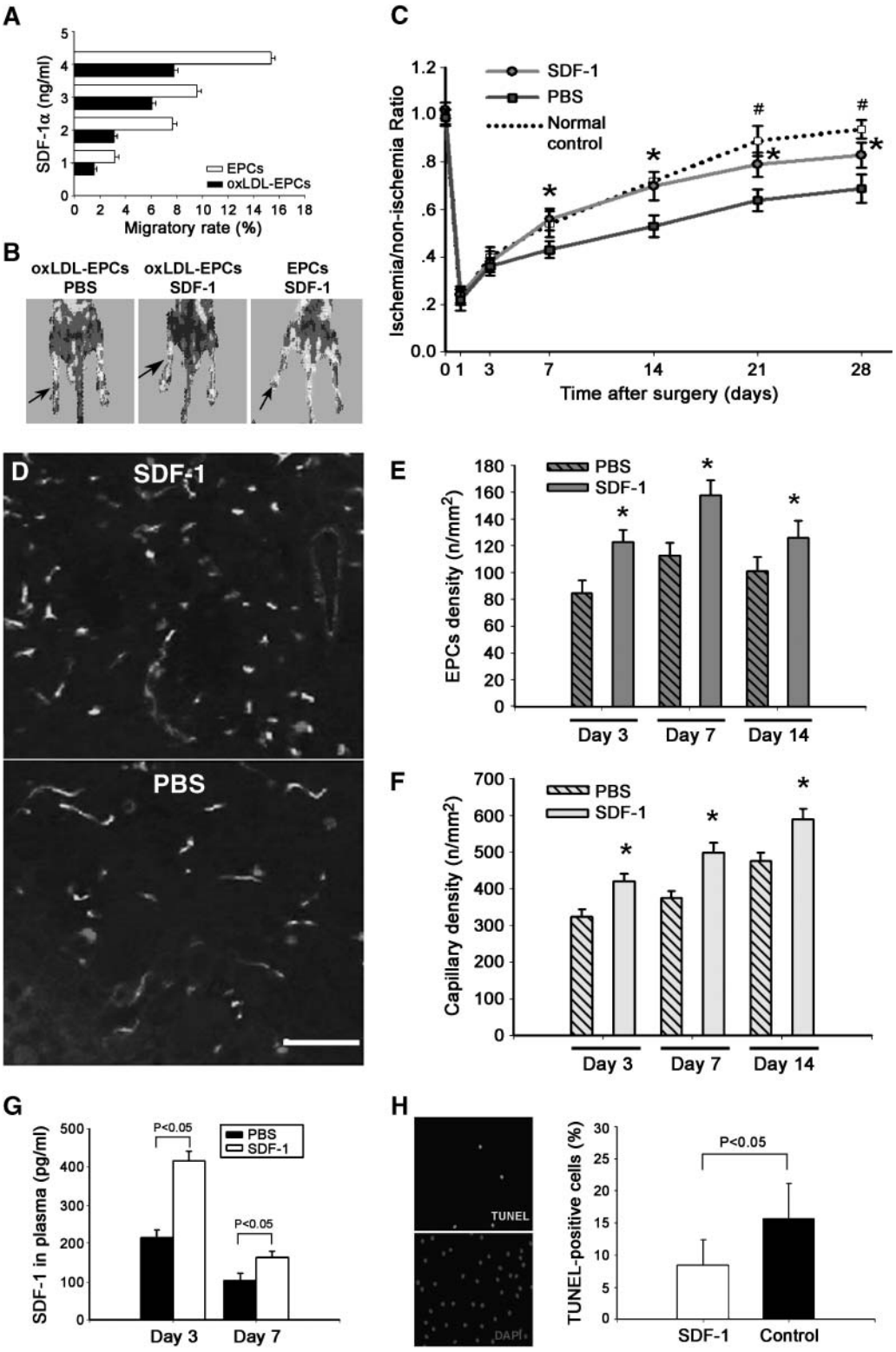

Fig. 4. Stromal cell-derived factor (SDF-1) treatment ameliorated the defect. A: SDF-1 improved the chemoattractant effect of OxLDL-EPCs. Normal EPCs were used as a control. B: Representative LDPI of PBS- or SDF-1-treated limbs after OxLDL-EPC transplantation. Normal EPCs were used as a control. C: Quantitative analysis of perfusion recovery as measured by the ischemia-nonischemia perfusion ratio at different time points (n = 15; * $P < 0.05$). D: Representative photomicrographs of double fluorescence labeling in ischemic muscles: transplanted Dil-labeled OxLDL-EPCs in red, and host mouse vasculature in green. Bar = 50 μm. E: Quantitative analysis of incorporated EPCs. The density of Dil-labeled OxLDL-EPCs (red fluorescence) in tissue sections of ischemic muscles was greater in the SDF-1 treatment group than in the PBS group at days 3, 7, and 14 (* $P < 0.05$). F: Quantitative analysis of host capillary density. The density of murine ECs (green fluorescence) was greater in the SDF-1 treatment group than in the PBS group at days 3, 7, and 14 (* $P < 0.05$). G: Circulating SDF-1 level of ischemia tissue from local injection of SDF-1 or PBS. H: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay of the apoptotic EPCs after SDF-1 in vitro treatment. White indicates TUNEL-positive cells, and grey indicates nuclei (DAPI-stained) (n = 5). For A, C, and E–H, results are expressed as means ± SEM.

fewer OxLDL-EPCs were recruited to the ischemic sites in vivo and migrated in response to VEGF in vitro. Thus, transplantation of OxLDL-EPCs was less effective at augmenting capillary density, blood perfusion, and functional recovery of the ischemic hindlimbs. These data provide evidence that the ultimate degree of physiological improvement is critically dependent on sufficient EPC recruitment at an early time point, which was impaired in OxLDL-EPCs.

Previous data have suggested that EPCs work in concert with existing ECs to form vessels rather than forming entirely new vessels de novo (23). In this study, we demonstrated the incorporation and/or entrapment of transplanted EPCs in the vasculature or the perivascular region in vivo and their participation in tubule formation in vitro. The impaired ability of OxLDL-EPCs to incorporate into tubules with HUVECs suggests that less vessel network formation contributed by OxLDL-EPCs occurs in vivo. Because tubule formation on matrigel is a global assay evaluating multiple cellular processes involved in blood vessel growth (23), it is likely that other unexamined components of blood vessel growth may be impaired in OxLDL-EPCs.

Finally, we demonstrated that local intramuscular delivery of SDF-1 restored the therapeutic efficacy of OxLDL-EPCs by recruiting more OxLDL-EPCs to ischemic sites. From these data, we found that the reduced recruitment of OxLDL-EPCs was attributable to both decreased expression of CXCR4 and increased apoptosis. It is known that SDF-1 plays an important role in ischemia-induced trafficking of stem cells from peripheral blood to ischemic tissues and also in the entrapment of circulating cells in the perivascular area (24). Our results indicate that a great number of OxLDL-EPCs were recruited to the SDF-1-treated ischemic tissue and promoted ischemia recovery. This is because OxLDL-EPCs express CXCR4, a receptor of SDF-1, albeit less than control cells. Although less CXCR4 was expressed on OxLDL-EPCs, they still responded to SDF-1, and injection of SDF-1 locally greatly enhanced the number of recruited OxLDL-EPCs. This suggested that other mechanisms played an important role in blood flow recovery. The restored therapeutic efficacy may also be related to an additional contribution of upregulated VEGF expression after SDF-1 administration, as reported by Yamaguchi et al. (20), as well as by augmentation of circulating SDF-1 levels (Fig. 4G). Increased plasma SDF-1 can strengthen the mobilization of stem/progenitor cells from bone marrow (21), which may account for improved recovery. In addition, SDF-1 administration could enhance Akt and endothelial nitric oxide synthase activities, which augment therapeutic neovascularization (25). It also seems likely that, in addition to transplanted OxLDL-EPCs, SDF-1 might stimulate host ECs from preexisting blood vessels and host EPCs derived from bone marrow.

There are some limitations to our study. First, the ex vivo culture procedure might ameliorate or exacerbate the influence of the OxLDL on the function of EPCs. However, we were unable to perform direct measurement of EPCs from patients with high serum OxLDL, because these patients with hypercholesterolemia usually also have diseases such as hypertension, diabetes mellitus, and cardiovascular diseases, which all influence EPC kinetics (26). It has also been reported that the levels of circulating OxLDL were significantly higher in patients with cardiac events than in patients without (27). We were unable to exclusively investigate the sole impact of OxLDL on patient EPCs; therefore, we chose to investigate the effect of OxLDL on ex vivo expanded normal EPCs so as to closely resemble the in vivo conditions in which EPCs are influenced by increased OxLDL. Second, as OxLDL in patient plasma might be lower in concentration but longer in duration of influence toward EPCs, our study did not fully resemble the in vivo situation. Third, because OxLDL decreased the proliferation (11) and increased the apoptosis (12) of EPCs, there remains the possibility that, in addition to impaired recruitment, impaired therapeutic vasculogenesis was also attributable to the attenuated survival and increased apoptosis of OxLDL-EPCs in vivo.

To the best of our knowledge, this study represents the first direct proof of the impaired therapeutic potential of OxLDL-EPCs in ischemia-induced neovascularization. This defect was through an array of cellular processes: impaired homing and migration as a result of less expression of CXCR4 and apoptosis, reduced adhesion, and attenuated ability in the incorporation and/or entrapment into the vasculature and the perivascular region. Further studies are necessary to confirm these results and to assess the exact mechanisms of altered activity in OxLDL-EPCs and of SDF-1-related effects.

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