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ORIGINAL ARTICLE

Autologous endothelial progenitor cells transplantation promoting endothelial recovery in mice

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Summary

Transplantation of endothelial progenitor cells (EPCs) restores endothelial function. The present study was designed to determine the effect of autologous EPCs transplantation on the regeneration of endothelium in mice. Mice splenectomy was performed 14 days before carotid artery injury, and mononuclear cells were isolated and cultured in endothelial growth media for 7 days. EPCs were confirmed by immunostaining (CD31, endothelial nitric oxide synthase (eNOS) and double positive for 1,1'dioctadecyl-3,3,3',3-tetramethylindocarbocyanine (DiI)-low-density lipoprotein and ulex europaeus agglutinin (UEA)). Cell counts and fluorescence-activated cell sorting for stem cell marker were performed. 1×10^6 4-,6-Diamidino-2-phenylindole- labeled EPCs or saline were injected through tail vein after wire injury. Two weeks after transplantation, cell tracking and immunohistochemical staining showed homing and incorporation of labeled EPCs in injury artery. Administration of EPCs enhanced reendothelialization ($P < 0.05$) after 1 week and inhibition of neointima formation at 3 weeks compared with that of saline ($P < 0.05$, $n = 6$). These data demonstrate that delivery of autologous EPCs is associated with accelerated reendothelialization and reduced neointimal formation. Thus, delivery of autologous EPCs represents an important vasculoprotective approach to attenuate the response to acute vascular injury.

Introduction

Endothelial progenitor cells (EPCs) have potential to incorporate into the site of vessel injury and differentiate into endothelial cells, therefore, contributing to improvement of endothelial function. At present, EPCs transplantation has become a novel strategy in recovery of impaired endothelium, which is associated with accelerated reendothelialization or reduced neointimal formation [1–6]. Most of these previous reports considered autologous transplantation for its substantial superiority and indicated a potential therapeutic method for actual clinical applications.

Mouse models mimicking human diseases are more and more popular especially for their advantages in gene therapy. It is now clear that similar genes and signaling pathways regulate the development of the heart and vasculature in mice and humans [7]. Hence, studies on mouse models are extremely important tools in trying to understand the mechanisms and improve cardiovascular diseases therapy. However, there is no report about autologous EPCs applications in mice carotid artery injury, probably for less EPCs in peripheral blood and bone marrow extract.

Spleen is an important hematopoietic organ during embryonic period, which also preserve haemopoietic ability after birth in mice. Multiple studies reported that spleen-derived mononuclear cells (MNCs) in endothelial-specific medium can be differentiated into EPCs [6,8–12]. Recently, more and more studies are being done on spleen-derived EPCs [6,8–17], and indicated that spleen-derived EPCs may be an important tool in stem cell research.

In the current study, we investigated the effects of autologous transplantation of spleen-derived EPCs on the recovery of injury carotid artery in mice.

Materials and methods

Splenectomy

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Third Military Medical University and followed with Principles of Laboratory animal care (NIH publication, Vol 25, No. 28, August 16, revised 1996). C57BL/6 mice (Institute of Zoology of Daping Hospital, China) were anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally. After 2–10 min, plantar reflexes disappeared. A 1.5–2-cm incision was made at the left hypochondrium. The spleen was exposed and the artery and efferent venule were tied off. After the spleen was removed, animals' abdomen was closed and sutured. Animals were allowed to recovery for 14 days before carotid arterial injury.

MNCs isolation and culture

Endothelial progenitor cells were cultured according to previously described techniques [6,8–17]. Briefly, homogenized splenic tissue was mechanically minced and MNCs were isolated by density gradient centrifugation with Ficoll separating solution. Cells were plated on fibronectin-coated 24-well plates($1 \times 10^6/cm^2$) in 0.5 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal-calf serum, vascular endothelial growth factor (VEGF, 50 ng/ml), basic fibroblast growth factor (bFGF, 5 ng/ml), epidermal growth factor (EGF, 10 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Four days after culture, nonadherent cells were removed by thorough washing with phospahte-buffered saline (PBS). The medium was changed every 3 days.

In order to compare the amount of spleen-derived cells, bone marrow MNCs were flushed from the femurs and tibias with Hanks' medium and cultured according to above methods. Cell count including bone marrow and spleen MNCs and EPCs were performed using hemacytometer.

Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) was performed for detecting the haematopoiesis cell phenotype [18]. MNCs (3×10^5/100 μl) obtained from spleen or bone marrow were incubated with Fluorescein isothiocyanate (FITC)-conjugated anti-stem cell antigen 1 (Sca-1) (D7; Ebioscience, San Diego, CA, USA) for 30 min at 37 °C. After treatment, cells were fixed in 1% paraformaldehyde (PFA). Isotype-identical antibodies served as controls. Quantitative FACS was performed on a FACS flow cytometer (Becton Dickinson, San Jose, CA, USA) and the software (CellQuest, San Jose, CA, USA).

EPCs Characterization

After 7 days culture, MNCs were incubated with 2.4 μg/ml DiI–labeled acetylated low-density lipoprotein (LDL, Molecular Prob, Eugene, OR, USA) at 37 °C for 1 h. Then cells were washed with PBS for three times and fixed with 2% PFA for 10 min. After washed with PBS, the cells were reacted with FITC-labeled lectin from ulex europaeus agglutinin (UEA, vector Laboratories Inc. Burlingame, CA, USA) for 1 h. Samples were washed with PBS and viewed with an inverted fluorescent microscope and further demonstrated by a laser scanning confocal microscope (LSCM, Leica, Wetzlar, Germany). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs [6, 8– 12] ($n = 5$). Negative control was incubated with PBS instead of DiI-LDL and UEA.

For characterization of endothelium markers, immunofluorescence was performed using rabbit polyclonal antibodies against CD31, endothelial nitric oxide synthase (eNOS) (Santa cruz, Delaware, CA, USA). Briefly, cells were washed with cold PBS three times, and fixed with 2% PFA for 15 min. The cells were washed again with PBS for 3×5 min, 3% bovine serum albumin (BSA) in PBS were added and left for 30 min. Then, cells were incubated for 1 h at room temperature with CD31, eNOS diluted 1:500 with 3% BSA in PBS. After washing three times with PBS on a shaker for 10 min, the cells were exposed to goat anti-rabbit Rhodamine (TRITC)-conjugated antibody (1:1000) for 1 h in the dark. Nuclear staining with DAPI (4-,6-diamidino-2-phenylindole, Roche, Basel, Switzerland) for eNOS and CD31-positive EPCs mounting were performed ($n = 5$). Negative control was incubated with PBS instead of primary antibody.

EPCs labeling and detection *in vivo*

To track the homing of EPCs in the carotid artery, a DAPI dye (Roche) was used. Briefly, spleen-derived EPCs were digested with 0.25% trypsin and re-suspended at 1×10^6/ml in DMEM containing DAPI (10 μg/ml) for 20 min at 37 °C and then washed three times with PBS. DAPI staining was confirmed by inverted fluorescent microscope. Then, EPCs were resuspended in 200 μl saline for tail vein injection.

Animals were anesthetized 2 weeks after cell transplantation with an overdose of sodium pentobarbital. A incision was made on the neck. Both carotids were exposed and excised. After washed with PBS, blood vessles were embedded in optimal cutting temperature (OCT) and 7-μm cross-sections were examined under fluorescence microscopy for detection of DAPI-labeled cells.

In order to identify the specific endothelial cell (EC) marker, immunohistochemical staining was performed.

Briefly, sections were fixed in 2% PFA, blocked with 5% normal goat serum, and incubated with rabbit anti-mouse CD31 antibodies (1:500). Sections were then incubated with FITC-conjugated goat anti-rabbit secondary antibody.

Carotid artery injury and autologous EPCs Transplantation

Carotid artery injury [19] was performed 14 days after splenectomy. Heparin (300 U/kg) was intraperitoneally administrated. After 30 min, animals were anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally. Left carotid arteries were exposed through a midline incision to the neck. 6-0 silk sutures were placed around the common carotid and internal and external carotid arteries to temporarily restrict blood flow to the area of surgical manipulation. The artery was injured with a 0.014-inch-diameter flexible angioplasty wire. The wire was advanced and withdrawn three times to ensure reliable effect. The total length of denuded common carotid artery was 5 mm from the bifurcation of carotid arteries in all animals. Then, the external carotid artery was permanently ligated. The temporary ligatures were released to allow blood flow to be restored through the internal carotid artery. The skin was closed with single sutures using 6/0 silk. Labeling cells (1×10^6) in 200 μl saline or saline alone (control group) were injected through tail vein ($n = 6$).

To confirm endothelial denudation of the carotid artery injury, mice were killed 10 min after the denuding procedure. Perfusion fixation was carried out using 2.5% glutaraldehyde. The vessels were cut open longitudinally and dehydrated through ethanol. After sputter-coated with gold/palladium, the specimens were examined with scanning electron microscopy (Amray 1000B, MA, USA).

Assessment of reendothelialization

One week after carotid artery injury and labeled EPCs transplantation, 200 μl of 5% Evans blue diluted with saline was injected into the heart with a 27-gauge needle. Then, 4% PFA was administrated for perfusion fixation. The left common carotid artery was then harvested 5 mm from the carotid bifurcation. After that, the artery was opened longitudinally and placed on slides. The areas unstained in blue and the total carotid artery areas were measured. The ratio of areas stain in white/the total carotid artery area were calculated ($n = 6$).

Measurement of medial and neointimal area

Three weeks after carotid artery injury, carotid arteries were embedded in paraffin after perfusion fixation with 2% PFA. Cross-sections (5 μm) were stained with hematoxylin–eosin according to standard protocols. All sections were examined under an inverted microscope. Morphometric analysis, including medial and neointimal area measurement, were performed with the use of Image-Pro Plus 5.1 (Media Cybernetics Inc. Bethesda, MD, USA) ($n = 6$). Endoluminal, internal elastic laminar, and external elastic laminar borders were manually traced with software (Image ProPlus) used to calculate intimal and medial areas.

Statistical analysis

All values were expressed as mean ± SD. One-way ANOVA was performed for comparison of two measurements. A probability value of $P < 0.05$ was considered to denote statistical significance.

Results

Cells count of spleen and bone marrow

In order to determine the optimal source of EPCs, we evaluated the phenotype and number of bone marrow and spleen MNCs. Interestingly, Sca-1-positive cells were higher (Fig. 1a–c) in spleen ($n = 4$) than bone marrow ($n = 5$), which indicated less mature and stronger proliferation of spleen MNCs. Furthermore, in terms of overall yield, greater number of cells were recovered from the spleen than combined femurs and tibias (4.32×10^7 spleen and1.48×10^7 femurs and tibias, Fig. 1d, $n = 6$). Even after 14 days cultured, more differentiated EPCs were obtained from spleen than from bone marrow ($0.71 \pm 0.26 \times 106/2$ femurs and tibias; $1.29 \pm 0.32 \times 106$ /spleen, Fig. 1e, $n = 6$), which may be helpful for autologous transplantation. Therefore, in terms of both yield and stem cell marker staining, the spleen is the preferred source of EPCs.

Characterization of EPCs

Endothelial progenitor cells were thought as a subtype of hematopoietic progenitor cells showing endothelial cell feature. To test the differentiation of MNCs towards endothelial cells, we harvested and cultured MNCs from spleen. After 7 days culture under endothelial-condition, MNCs appeared to be clone like and resulted in a spindle-shaped, EC-like morphology (Fig. 2a). The center cells in clone show less incorporation of DiI-LDL (red) in which implied outer cells were differentiating gradually to endothelial cells (Fig. 2a). Next, we tested whether the adherent cells would take up LDL-cholesterol particles from the media and stained positive for UEA-lectin (Fig. 2b), which were widely used to define EPCs of hematopoietic origin. LSCM showed high double positive of cultured MNCs (yellow, 87.32 ± 5.13%, Fig. 2b, $n = 5$) permiting the characterization of endothelial

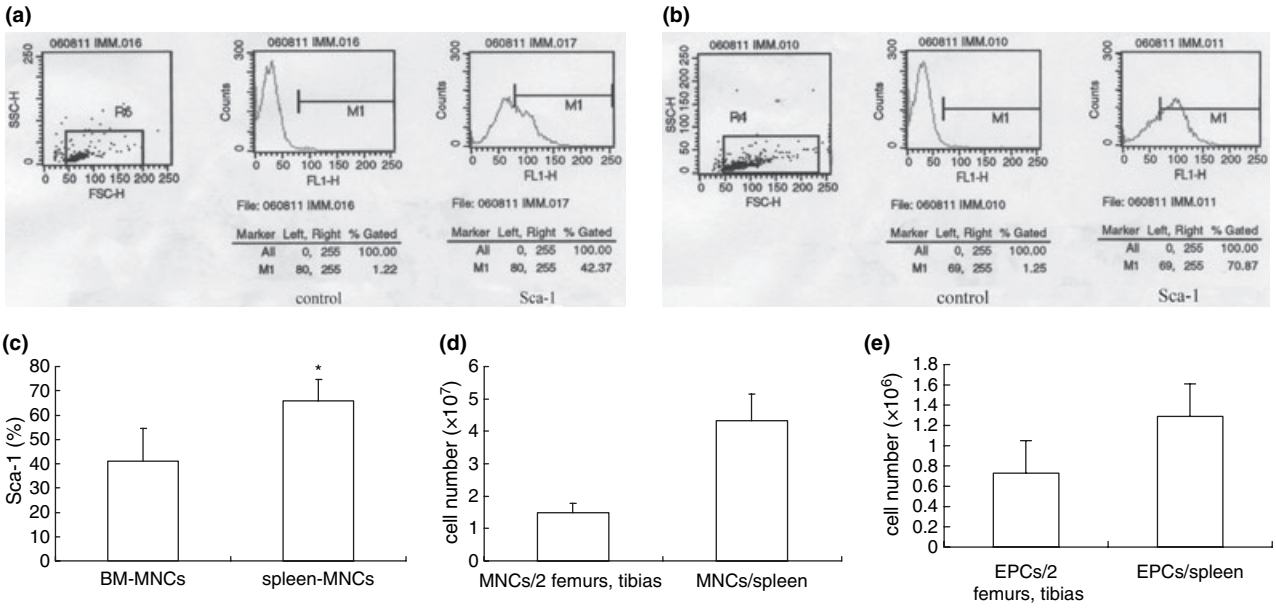

Figure 1 Representative 4-quadrant fluorescence-activated cell sorting (FACS) analysis of bone marrow-mononuclear cells (MNCs) (a, $n = 5$) and spleen-MNCs (b, $n = 4$) for FITC-stem cell antigen 1 (Sca-1), and quantification (mean ± SD) of MNCs in bone marrow and spleen (c). $*P < 0.05$ vs. BM-MNCs. Number of MNCs isolated from spleen and femurs and tibias ($\times 10^7$) (d), after 14 days culture, EPCs ($\times 10^6$) were counted (e, $n = 6$).

function. Furthermore, CD31and eNOS staining were performed for detecting endothelial phenotype. Immunohistochemistry indicated most cultured MNCs expressed CD31 (89.9 ± 6.16%) and eNOS (92.4 ± 1.77%) (Fig. 2c and d, $n = 4$). Most of the positive signal were located in plasma. So, different characterization of endothelial cell including cell morphology, biological function and phenotype indicated that they were differentiated endothelial cells.

4-,6-Diamidino-2-phenylindole staining showed nuclear dying that was helpful to calculate the cell numbers and fluorescence location. In addition, this method is effective to exclude interference of noncell substance under fluorescence background.

Reendothelialization of denuded carotid artery

The key features of the carotid artery injury model are determined by whether all the endothelial cells were removed from these arteries. Scanning electron microscopy was commonly used to examine the surface of the vessel. In our study, the carotid artery injury was confirmed by scanning electron microscopy 10 min after the denudation. As shown in Fig. 3a, no endothelial cells were observed on the surface of artery. Also there was some platelet-like substance (white arrows) that covered the sub-endothelial matrix. Compared with injured group, uninjured group showed monolayer of endothelial cells (Fig. 3b).

Reendothelialization of the denuded vessels is an important step for recovery of carotid artery injury. Traditionally, 5% Evans Blue solution was used to mark de-endothelialized area of the carotid artery. To clarify the effect of spleen-derived EPCs transplantation on endothelium injury, the mice were injected with Evans Blue 7 days after operation. Denuded (blue) and reendothelialized (white) area were identified. Reendothelialization are represented by the ratio of white area/total area. Endothelial cell regeneration was significantly enhanced in the spleen-EPCs-treated vessels than the vessels treated with saline injection (65.8 ± 3.9% vs. 40.3 ± 1.9%, $P < 0.05$, Fig. 3c–e, $n = 6$). The data clearly demonstrated that EPCs delivery markedly enhanced reendothelialization of denuded carotid artery.

Inhibition of neointimal hyperplasia

Neointimal hyperplasia leads to in-stent restenosis and subsequently tissue ischemia. Mobilization and transplantation of EPCs had been novel strategy for vascular stenosis because of rapid and complete reendothelialization. In order to determine the effect of EPCs transplantation on vascular lesion formation, neointima was evaluated through Image ProPlus. Because the diameter of the carotid artery is variable, neointimal thickness was assessed in terms of intima-to-media area ratios.

Only monolayer endothelial cells were seen along the inner lumina of carotid artery, which indicated no

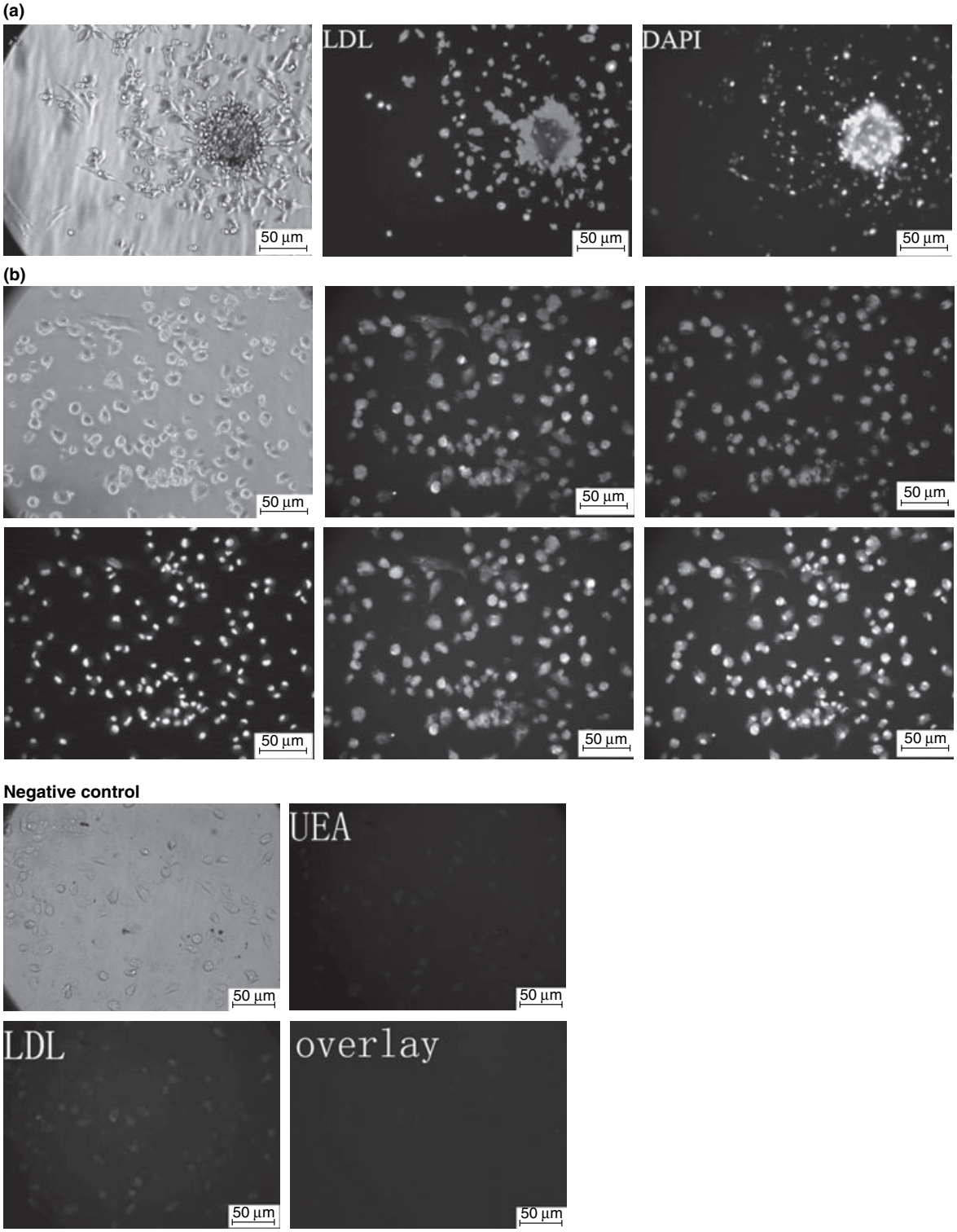

Figure 2 Mononuclear cells (MNCs) derived from spleen differentiated into cells with endothelial progenitor cell (EPC) phenotype under EPCs-specific conditions. Culture of mononuclear cells resulted in the emergence of colonies and characteristic spindle-shaped EPCs (a), characterization of EPCs by immunofluorescent stainings was performed. Cells double positive for DiI-LDL uptake and lectin binding were identified as EPCs (b, $n = 5$), which expressed CD31 and eNOS (c and d, $n = 4$) too. Cell counting was documented with corresponding nuclear staining with 4-,6-diamidino-2-phenylindole (DAPI). Scan bar = 50 μm. Magnification 400×.

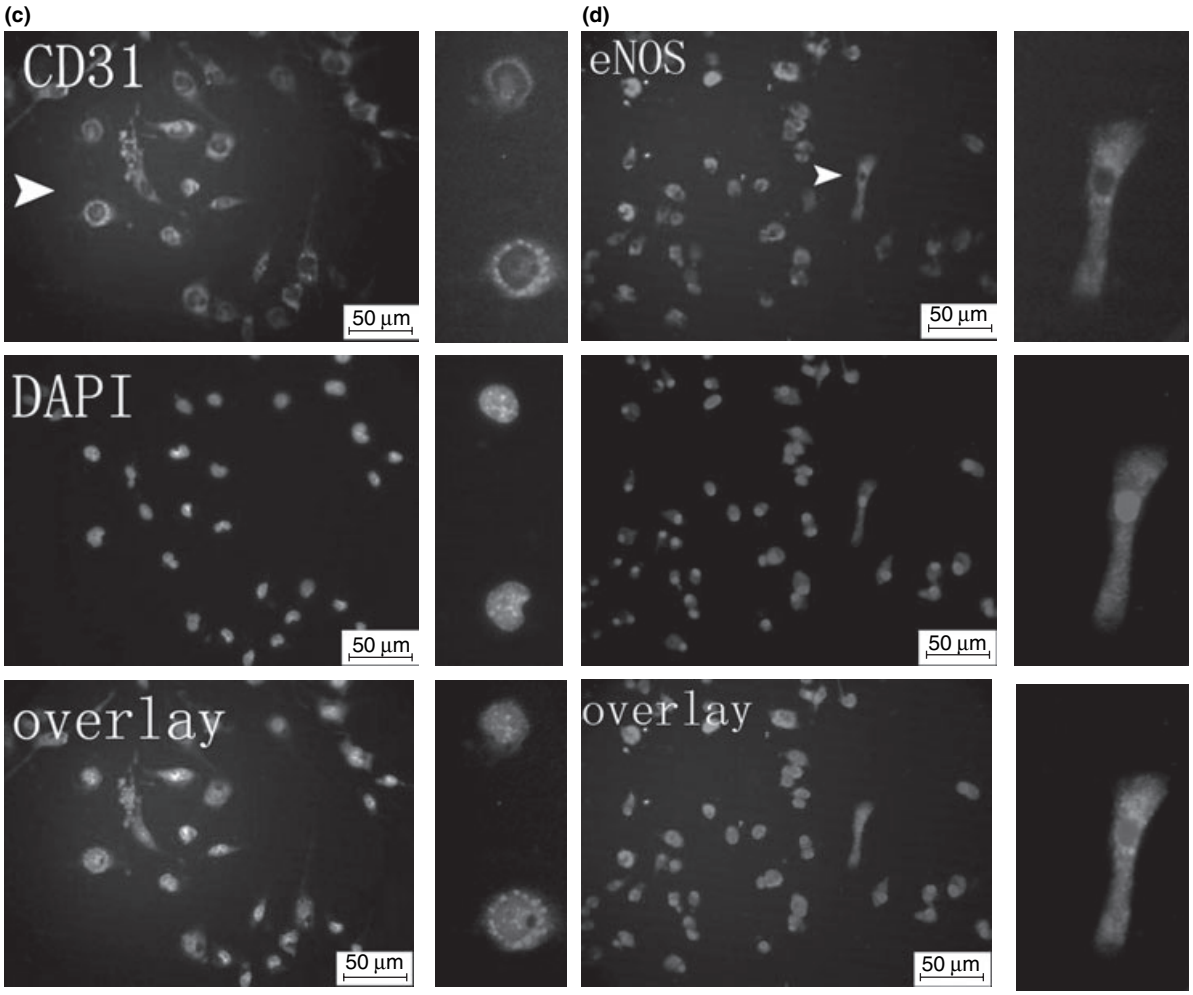

Figure 2 Continued

evidence of neointima in uninjured vessels (Fig. 4a). However, wire injury resulted in a prominent neointima (between two black arrows) in the arteries of saline-treated animals (Fig. 4b). Transplantation of EPCs reduced neointima development in the injured vessels (Fig. 4c). Compared with the saline control, morphometric analysis showed a decrease of neointima/media ratio in the EPCs-transplanted mice (0.34 ± 0.045 vs. 0.74 ± 0.181, $P < 0.05$, Fig. 4d, $n = 6$). These results suggested that transplantation of EPCs contribute to inhibition of neointimal hyperplasia.

Labeled EPCs are detectable in injured artery

To determine whether labeled autologues EPCs incorporated in the injured vessel wall and differentiated into endothelial cells, animals received 1×10^6 autologous DAPI-labeled EPCs by tail vein injection after carotid injury. Two weeks later, EPCs tracking and immunohistochemistry were performed. Autologous DAPI-labeled EPCs were identified as blue fluorescence cells within the neointima (Fig. 5a). No labeled cells were identified in uninjured control arteries. Labeled cells were seen lining the lumen that co-stained for endothelial markers CD31 (Fig. 5b green). This revealed that EPCs could home to injury sites where they function to promote recovery of injured blood vessel.

Discussion

In the present study, we demonstrate, for the first time, the therapeutic potential and technical feasibility of transplantation of autologous spleen-EPCs in the setting of carotid artery injury.

Strategies that enhance the number of EPCs may facilitate angiogenesis and recovery of injured endothelium. It has been described that both peripheral blood and bone marrow could be used as a source for endothelial

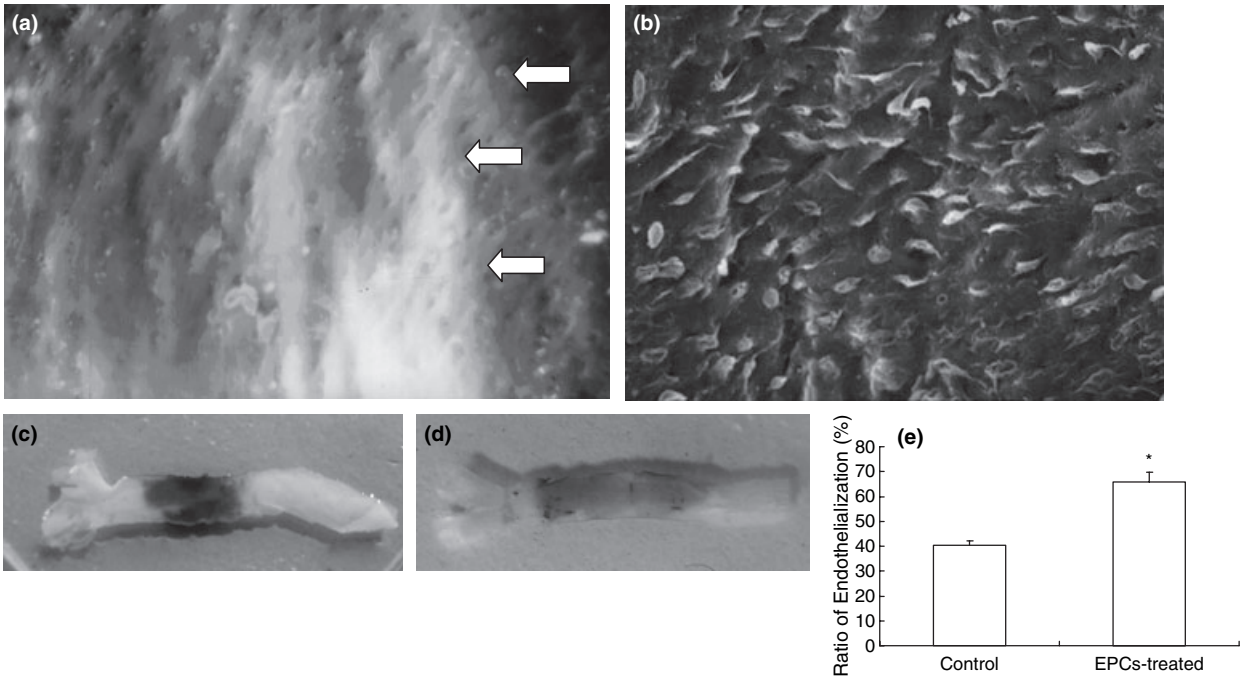

Figure 3 Scanning electron micrograph of a mouse carotid artery injury (a) 10 min after denudation. Compared with uninjured carotid artery (b), complete endothelial denudation was achieved. Scan bar = 100 μm, Magnification 600×. Evans blue staining identified segments of denuded and reendothelialized (white) surfaces. Compared with placebo group (d), endothelial progenitor cell (EPCs)-treated arteries (c) showed significantly recovery of endothelium (e), $*P < 0.05$ vs. control, $n = 6$.

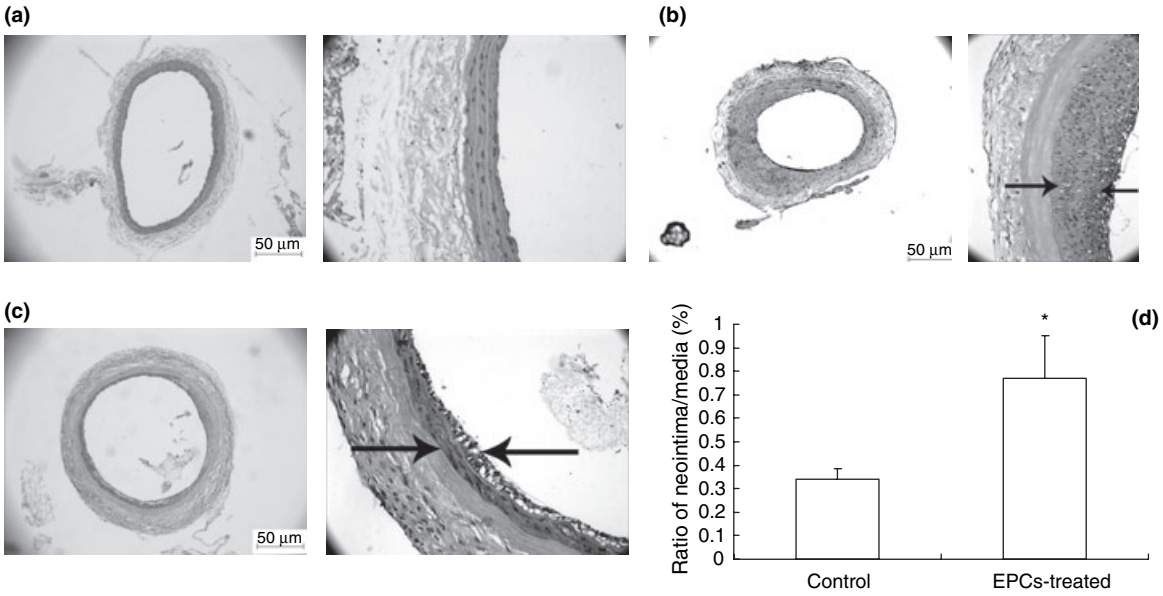

Figure 4 Inhibition of neointimal proliferation by endothelial progenitor cells (EPCs) transplantation in injured carotid arteries. Scan bar = 50 μm. Magnification 400× (left). Uninjured artery (a), saline-injected injured artery (b), injured artery transplanted with EPCs (c), neointima/media ratios in saline-treated and EPC-transplanted injured vessels (d). *EPCs-treated vs. saline, $P < 0.05$, $n = 6$.

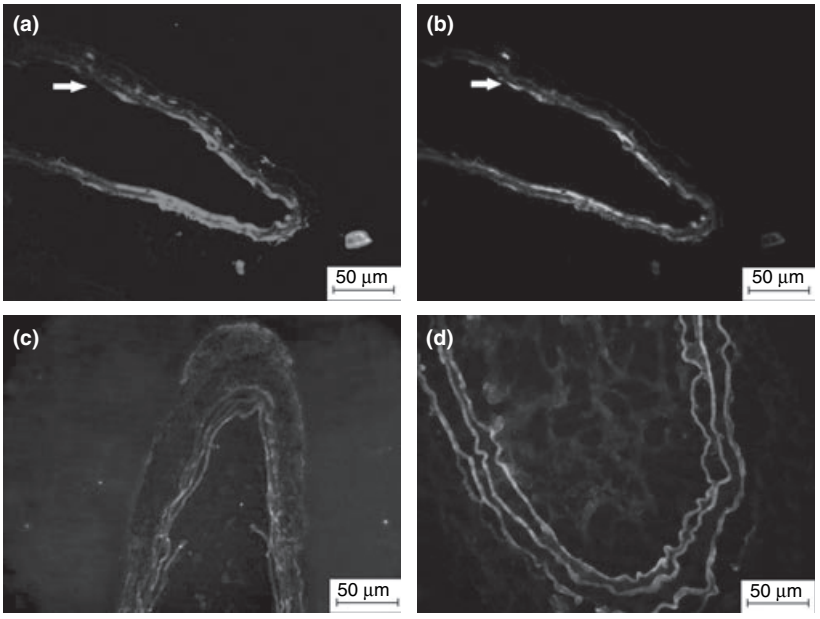

Figure 5 Endothelial progenitor cells (EPCs) homed to the vascular injury site after transplantation. Homing of transfused cells was strictly restricted to the deendothelialized area (a), immunohistochemical detection of CD31 showed endothelial phenotype in frozen sections from vessel transplanted (b), control of DAPI and CD31 showed only background autofluorescence in the elastic fibers (c, d). However, no staining cell was observed. Scale bar = 50 μm, Magnification 400×.

precursor cells. But, it is difficult to obtain enough cells (1×10^6) for autologous transplantation in mice. Spleen-derived MNCs can trans-differentiate into endothelial cells and provide another useful method for stem cell study [6,8–12]. At present, spleen is considered as a major reservior of EPCs that play an important role during early and later EPCs mobilization [6,16,17]. These lines of evidence indicate that spleen may be a potential provider of stem cells for cardiovascular disease.

Stem cell antigen 1 (Ly-6A/E) is a recognized haemopoietic stem cell (HSC) marker in mice and Sca-1^+ HSCs can be found in the adult bone marrow, fetal liver and mobilized peripheral blood and spleen. In this present study, we found that there are enough MNCs for further culture and transplant in spleen. Interestingly, spleen-derived MNCs showed high levels of Sca-1 expression than bone marrow indicating higher differentiation potential into endothelial cells. This may be attributed to high proportion of monocytes and macrophages [20] in spleen-derived MNCs. Therefore, spleen may be a suitable source of EPCs in mice. However, it need to be confirmed by other studies.

Endothelial progenitor cells have been a focus of cardiovascular study. However, there is still no accepted standard method or criteria for definition. In our study, incorporation of acetylated LDL/binding of lectins was used for characterization of endothelial function. CD31 and eNOS stainings were performed for detecting endothelial phenotype. Moreover, it was further confirmed by spindle shaped morphology. Our data showed cultured MNCs had typical features for endothelial cells.

Endothelial progenitor cells play important roles in angiogenesis and in the repair of injured endothelium. Both Gulati *et al*. and Griese *et al*. [3,5] reported that the delivery of autologous culture-modified mononuclear cells can accelerate reendothelialization, reduce neointimal formation in rabbits, which may be partly attributed to the release of proangiogenic cytokines from EPCs [1]. A similar strategy applied in rat demonstrated that seeding of bone marrow-derived EPCs resulted in rapid repopulation of the denuded artery and indicated genetically engineered EPCs can serve as cellular vehicles for targeted vascular gene transfer [2]. Kong *et al*. showed that autologous rabbits EPCs transplantation effectively prevents thrombosis and reduces neointimal hyperplasia in denuded carotid arteries by promoting reendothelialization. This effect was further enhanced by genetic engineering with eNOS [4]. These findings suggested that autologous EPCs-based therapies have beneficial effects on vascular structure and function.

Mice are widely used for their short gestation period, more litter size, and relatively low maintenance costs. Besides, more and more gene-targeted 'knockout' and transgenic over-expression experiments were performed using mice, rather than other animals [7]. At present, mouse models play critical roles in revolutionizing the ability to diagnose and treat patients with cardiovascular diseases [21]. Werner *et al*. attempted to inject freshly isolated MNCs and EPCs into mice with carotid artery injury. Interestingly, they found that neointima reduction was more prominent after intravenous transfusion of spleen-derived MNCs, which, they think, may be attributable to an increased transdifferentiation rate of MNCs to endothelial cells [6]. MNCs, as a mixed and undifferentiated cell population, show no adequate

marker and can avoid destruction by the host immune system. However, EPCs are not pluripotent, self-renewing stem cells, but rather lineage-committed progenitor cells. So, in our opinion, attenuated effects of EPCs may probably relate with immunologic rejection followed by allogeneic transplantation. Recently, Wassmann *et al.* reported that intravenous transfusion of spleen-derived MNCs improved endothelium-dependent vasodilation in atherosclerotic apoE(-/-) mice, indicating an important role of EPCs for the repair of ongoing vascular injury [8]. However, there is no report about autologous EPCs transplantation in mice. Our strategy was designed to overcome this inherent limitation of previous approaches that may benefit future application in humans.

We found EPCs transplantation increased reendothelialization of the denuded vessels and reduced neointima formation. On the other hand, labeling EPCs are located in the injured site, which also showed endothelial characteristic. Our data indicated that incorporation of autologous EPCs resulted in accelerated reendothelialization, which in turn is known to be associated with inhibition of smooth muscle cell proliferation [22], and reduced neointimal formation.

These results show that autologous spleen-derived EPCs transplantation has beneficial effect on endothelial regeneration and function after injury. Furthermore, this study provides new evidence of spleen significance in stem cell biology and therapy.

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Authorship

Performed research/wrote paper: XZ; Designed research: LH; analyzed data: YY; collected data: YF; contributed important reagents: YZ.

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