A Preliminary Study of the Therapeutic Role of Human Early Fetal Aorta-derived Endothelial Progenitor Cells in Inhibiting Carotid Artery Neointimal Hyperplasia

Rong-Wei Xu[1,2], Wen-Jian Zhang[3], Jian-Bin Zhang[1,2], Jian-Yan Wen[1], Meng Wang[3], Hong-Lin Liu[3], Lin Pan[3], Chang-An Yu[3], Jin-Ning Lou[3], Peng Liu[1,2]

[1]Department of Cardiovascular Surgery, China-Japan Friendship Hospital, Beijing 100029, China
[2]Graduate School of Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100073, China
[3]Institute of Clinical Medical Sciences, China-Japan Friendship Hospital, Beijing 100029, China

Abstract

Background: Endothelial cell damage is an important pathophysiological step of restenosis after angioplasty and stenting. Cell transplantation has great therapeutic potential for endothelial recovery. We investigated the effect of transplanting endothelial progenitor cells (EPCs) derived from human early fetal aortas in rat injured arteries.

Methods: The carotid arterial endothelium of Sprague-Dawley rats was damaged by dilatation with a 1.5 F balloon catheter, and then EPCs derived from human early fetal aortas (<14 weeks) were injected into the lumen of the injured artery in transplanted rats, with an equal volume of normal saline injected into control rats. Rats were sacrificed at 2 and 4 weeks after treatment and transplanted cells were identified by immunohistochemical staining with anti-human CD31 and anti-human mitochondria antibodies. Arterial cross-sections were analyzed by pathology, immunohistochemistry, and morphometry.

Results: Green fluorescence-labeled EPCs could be seen in the endovascular surface of balloon-injured vessels after transplantation. The intimal area and intimal/medial area ratio were significantly smaller in the transplanted group than in the control ($P < 0.05$) and the residual lumen area was larger ($P < 0.05$). After EPC transplantation, a complete vascular endothelial layer was formed, which was positive for human von Willebrand factor after immunohistochemical staining, and immunohistochemical staining revealed many CD31- and mitochondria-positive cells in the re-endothelialized endothelium with EPC transplantation but not control treatment.

Conclusion: EPCs derived from human early fetal aorta were successfully transplanted into injured vessels and might inhibit neointimal hyperplasia after vascular injury.

Key words: Carotid Artery Injury; Cell Transplantation; Endothelial Progenitor Cell; Human Fetal Aorta; Neointima

INTRODUCTION

Percutaneous transluminal angioplasty and stenting have been widely used for treating cardiovascular diseases, but it can also lead to endothelial injury, an important pathophysiological basis of restenosis after endovascular treatment.[1] Therefore, re-establishing a functional endothelium is important in inhibiting the neointimal proliferation.

Stem cell transplantation has been developed in recent years, and despite many unresolved problems, it has shown promising therapeutic potential. Endothelial progenitor cells (EPCs) are a special type of bone marrow-derived endothelial precursor cells that can enter into circulation after mobilization, home to the injured site and differentiate into endothelial cells (ECs).[2] Transplanting EPCs to repair vascular injury is a hot topic. However, EPCs isolated from bone marrow, peripheral blood, or cord blood are limited in number, for difficulties meeting the clinical requirement. Therefore, developing and establishing a safe and efficient method to isolate

Address for correspondence: Prof. Peng Liu,
Department of Cardiovascular Surgery, China-Japan Friendship Hospital,
Beijing 100029, China
E-Mail: liupeng61@126.com

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and culture humanized EPCs is important for the clinical therapeutic application.

In our previous study, we isolated EPCs from human early fetal aortas (<14 weeks) and established methods to induce EPC proliferation and differentiation into ECs *in vitro*.[3] Preliminary results showed that EPCs derived from early fetal aortas possessed the strong self-renewal capability and potential to differentiate into ECs, so these cells could be used for clinical treatment.[3] In this study, we investigated whether EPCs derived from human early fetal aortas could inhibit neointimal hyperplasia of injured vessels at 2 and 4 weeks after injury.

METHODS

Materials

All animal procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee and the experimental protocol was reviewed and approved by the Ethics Committee of China-Japan Friendship Hospital, Beijing, China. EPCs derived from human early fetal aortas (<14 weeks) were provided by Clinical Research Institute of China-Japan Friendship Hospital. Dulbecco's modified Eagle's medium (DMEM)/F12 medium, endothelial cell growth supplement (ECGS), leukemia inhibitory factor (LIF), gelatin, and trypsin were from Sigma (USA). Other materials included stem cell-tested fetal bovine serum (FBS, Biochrom, Germany), human epidermal growth factor (hEGF, Invitrogen, USA), basic fibroblast growth factor (bFGF, Invitrogen, USA), mouse anti-human CD31 monoclonal antibody (Chemicon, USA), mouse anti-human mitochondria monoclonal antibody (Millipore, USA), mouse anti-human von Willebrand factor (vWF) monoclonal antibody (Santa Cruz Biotechnology, USA), 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethyl ester (BCECF-AM) fluorescent dye (Invitrogen, USA), Evans blue dye (Wako Fine Chemicals, Japan), and 1.5 F balloon catheters (Boston Scientific, USA).

Cell culture

EPCs isolated from human early fetal aortas were maintained in DMEM/F12 medium containing 100 µg/ml ECGS, 5 ng/ml LIF, 20 ng/ml bFGF, 20 ng/ml hEGF and 10% FBS, in a humidified incubator (5% CO_2, 37°C). The medium was refreshed every 3 days and nonadherent cells were removed. Adherent cells were passaged every 3 days and the culture was maintained through days 7–10. Cells up to passage 3 were used for transplantation.

Animal experiments

Healthy purebred male Sprague-Dawley rats weighing 250–300 g were purchased from Vital River (Beijing, China). All rats underwent balloon injury of the left common carotid artery (CCA) as described.[4] Briefly, rats were anesthetized with 1% sodium pentobarbital (55 mg/kg, by intraperitoneal injection), then injected by tail vein with heparin sodium (100 U/kg) to prevent coagulation. The neck hair was removed. After conventional disinfection, an anterior midline incision was made, and the left CCA and internal and external carotid arteries were isolated. Two ligatures were placed proximally and distally around the external carotid artery. The distal ligature was tied off. After temporary occlusion of the proximal common and internal carotid artery with artery clips, a transverse arteriotomy was performed between the ligatures of the external carotid artery. A 1.5 F balloon catheter was inserted through the external carotid artery, inflated with 150 µl air, and passed along the vessel with rotation 3 times. After removal of the catheter, the proximal ligature of the external carotid artery was tied off. Normal blood flow was assured, and the skin was closed with single sutures using 5/0 silk. All rats were given intravenous heparin (100 U/kg) to prevent coagulation and intramuscular penicillin (2×10^5 U) to prevent infection postoperatively for 3 consecutive days [Figure 1].

### Microscopy of rat carotid artery endothelial denudation

At 30 min after balloon injury, 5% Evans blue dye (25 mg/kg) was injected intravenously, then 10 min later, the rats were sacrificed with an overdose of sodium pentobarbital and bilateral CCAs were removed and split longitudinally, washed with phosphate-buffered saline (PBS), then mounted and photographed by use of a stereoscopic microscope (M205 FA, Leica, Germany).

Transplantation regimen

The rats were divided into four groups: 2- and 4-week transplantation groups, and 2- and 4-week control groups (balloon injury only). The cell suspension (1×10^6 EPCs in 40 µl) was injected locally into the arterial lumen of the 2 transplantation groups, and the same amount of normal saline was injected into the arterial lumen of the 2 control groups. The proximal end of the external carotid artery was ligated before injection of EPCs or normal saline. After incubation of cells or saline for 30 min, the artery clips on the proximal common and internal carotid artery were released to restore blood flow. Rats were sacrificed at 2 or 4 weeks with an overdose of sodium pentobarbital.

Microscopy of progenitor cell adhesion and survival

Transplanted EPCs were incubated with 5 µl/ml BCECF-AM fluorescent dye in a humidified incubator (5% CO_2, 37°C) for 1 h. Then the cells were washed with PBS 3 times and injected into the injured carotid artery. At 24 h later, rats were sacrificed with an overdose of sodium pentobarbital. One centimeter of target vessel was excised and opened longitudinally. After washing with PBS, green
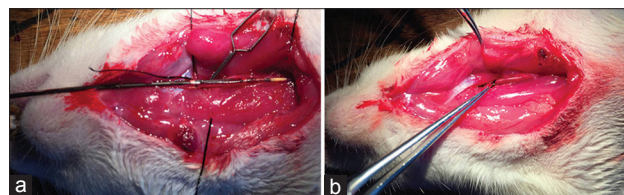
Figure 1: Establishment of rat carotid artery injury model. (a) Balloon injury (b) ligature blood vessel and resume blood flow.

fluorescence-labeled EPCs were identified by fluorescent stereoscopic microscopy (M205 FA, Leica).

Morphometric analysis

From transplanted and control rats sacrificed at 2 and 4 weeks after arterial injury, 1 cm of target vessel was excised and fixed in 10% formalin, dehydrated, embedded in paraffin and sectioned at various intervals. Four sections of each vessel were randomly selected for hematoxylin-eosin (HE) staining. The following indicators were calculated using the Image-Pro Plus (Media Cybernetics Inc., USA): Intimal area (IA), intimal/medial area (I/M) ratio, and residual lumen area (LA).

Immunohistochemistry of progenitor cell differentiation

Parts of target vessels were incubated according to manufacturers' instructions with the antibodies for human CD31 (1:100), human mitochondria (1:60), and human vWF (1:250) to observe positive cells and their distribution. The positive granules appeared as brown or brown-black particles distributed in the cytoplasm.
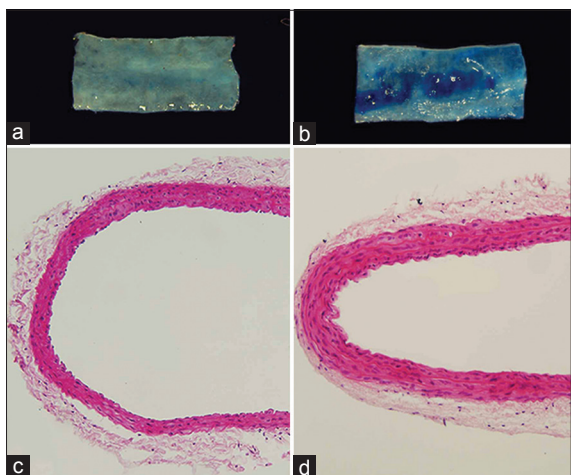
Statistical analysis

Statistical analysis involved the use of SPSS 19.0 (SPSS Inc., USA). Data are expressed as mean ± standard deviation (SD) and between-group comparisons involved unpaired Student's t-test. $P <0.05$ was considered statistically significant.

RESULTS

Evaluation of rat carotid artery balloon injury model

After balloon injury, the injured left CCA of rats showed Evans blue staining for denuded endothelium, with the uninjured right CCA not stained [Figure 2]. HE staining of the uninjured right CCA clearly showed each layer of normal CCA, with the integrated monolayer of ECs showing blue-stained nuclei on the endovascular surface. The intima and adventitia were clearly visible and complete. The media comprised 3 layers of neatly arranged elastic, annular membrane, and smooth muscle cells. However, in the injured left CCA, the intima was stripped and the monolayer of ECs was absent.

Adhesion and survival of endothelial progenitor cells at the intimal injury site

At 24 h after transplantation of fluorescence-labeled EPCs, scattered green fluorescence could be seen in the endovascular surface of balloon-injured CCA under fluorescent stereoscopic microscopy, which suggested that injected EPCs could adhere to the intimal injury site and survive [Figure 3].
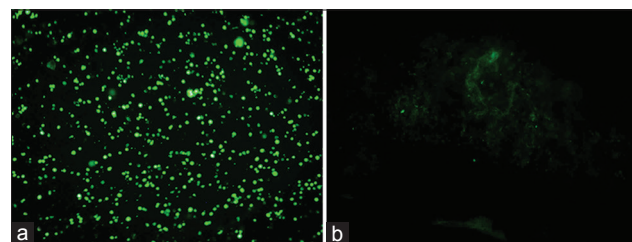
Effect of endothelial progenitor cells on neointimal hyperplasia and re-endothelialization of injured vessels

Each layer of the normal CCA was integrated without any intimal hyperplasia inside the lumen. At 2 and 4 weeks after balloon injury, the blood vessels from EPC-transplanted and control groups showed intimal hyperplasia, disordered and dense intimal and medial cells and narrowed lumen [Figure 4]. The IA and I/M ratio were lower in the transplanted group than in the control (both $P < 0.05$), and the LA was higher ($P < 0.05$) [Table 1]. Immunohistochemical staining for vWF, as a marker of vascular ECs, showed human vWF-positive cells distributed in the neointimal layer in the transplantation groups, which suggested that a complete vascular endothelial layer was formed [Figure 5].

Table 1: Comparison of intimal hyperplasia of the injured common carotid artery in rats between the endothelial progenitor cell transplantation and control groups after 2 and 4 week treatment

| Parameters	EPC transplantation (*n* = 5)	Control (*n* = 5)	*t*	*P*
2 weeks after treatment				
IA, mm²	0.069 ± 0.025	0.180 ± 0.063	2.845	0.047
I/M ratio	0.543 ± 0.145	1.603 ± 0.296	5.569	0.005
LA, mm²	0.262 ± 0.030	0.119 ± 0.021	−6.689	0.003
4 weeks after treatment				
IA, mm²	0.158 ± 0.031	0.277 ± 0.047	3.639	0.022
I/M ratio	1.315 ± 0.155	2.232 ± 0.159	7.149	0.002
LA, mm²	0.205 ± 0.071	0.081 ± 0.020	−2.948	0.042

Data are mean ± standard deviation. EPC: Endothelial progenitor cell; IA: Intimal area; I/M: Intimal/medial area; LA: Luminal area.

Figure 2: Evaluation of vascular balloon injury model. Normal blood vessels (a and c) and injured blood vessels (b and d) (a, b: Evans blue staining, original magnification, ×15; c, d: Hematoxylin-eosin staining, original magnification, ×200).

Figure 3: Adhesion and survival of endothelial progenitor cells at the intimal injury site (original magnification, ×100) (a) endothelial progenitor cells were labeled with green fluorescence (b) green fluorescence in the endovascular surface of injured vessel.
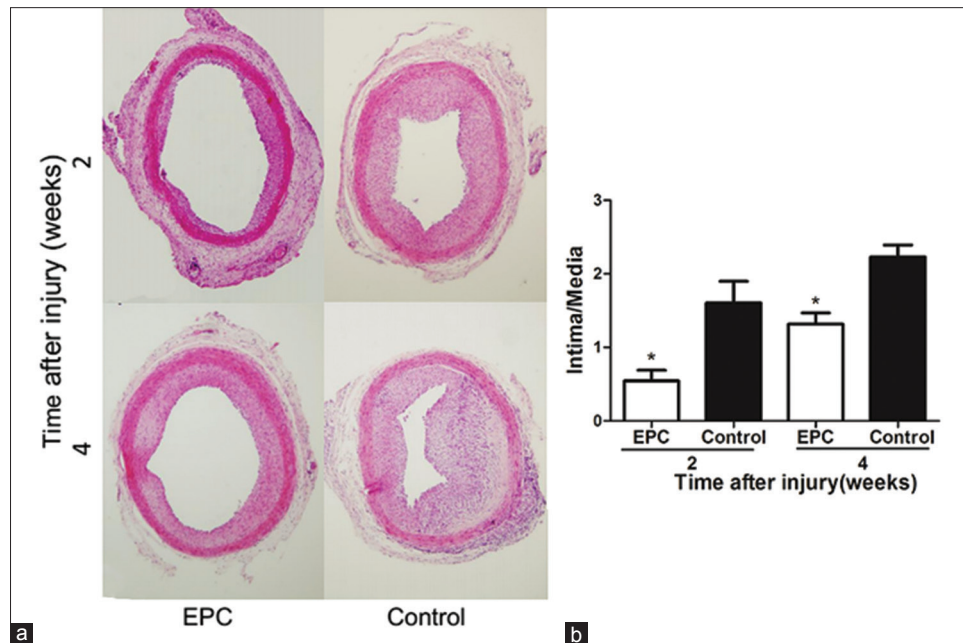
Figure 4: Inhibition of neointimal proliferation by endothelial progenitor cell transplantation in injured carotid arteries (a) representative photomicrographs of hematoxylin-eosin stained histological cross-sections in transplantation group ($n = 5$) versus control group ($n = 5$) at 2 and 4 weeks after carotid injury (original magnification, ×100) (b) intimal/medial area ratio was expressed as mean ± standard deviation. *$P < 0.05$ compared with controls. EPC: Endothelial progenitor cell.
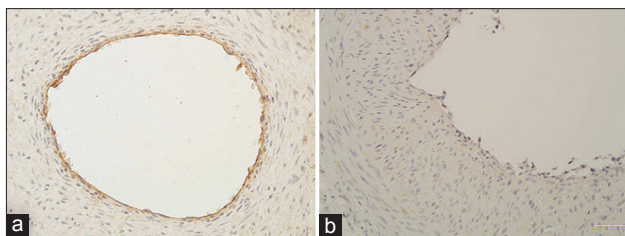
Figure 5: Re-endothelialization of injured vessel after endothelial progenitor cell transplantation or control treatment (human von Willebrand factor immunohistochemical staining, original magnification, ×400). Human von Willebrand factor staining in (a) transplantation group and (b) control group.

Identification of endothelial progenitor cells in the rat common carotid artery

Immunohistochemical staining showed many CD31- and mitochondria-positive cells distributed in the re-endothelialized endothelium in the transplantation group, which suggested that transplanted EPCs survived and differentiated into ECs in the injured vessels. However, control groups showed no CD31- and mitochondria-positive cells in injured vessels. Therefore, the human fetal aorta-derived EPCs were involved in the vascular repair and re-endothelialization [Figure 6].

DISCUSSION

EC damage is an important pathophysiological step of restenosis after angioplasty and stenting. Cell transplantation has therapeutic potential for endothelial recovery. We investigated the effect of transplanting EPCs from human early fetal aortas in rat injured carotid arteries.

Fluorescence-labeled EPCs could be seen in the endovascular surface of balloon-injured vessels after transplantation. The IA and I/M ratio were significantly smaller in the transplanted group than in the control group and the residual LA was larger. After EPC transplantation, a complete vascular endothelial layer was formed, which was positive for human vWF, and the re-endothelialized endothelium showed CD31- and mitochondria-positive cells with EPC transplantation. EPCs derived from human early fetal aortas could be successfully transplanted into injured vessels and may inhibit neointimal hyperplasia after vascular injury.

The rapid development of endovascular techniques and equipment has created a new era for the treatment of cardiovascular diseases. However, the postoperative restenosis of target vessels remains an intractable issue and a challenge for vascular biologists and interventional cardiologists. Studies have shown that restenosis after endovascular treatment represents an exaggerated healing response to iatrogenic injury of the vessel wall during angioplasty and stenting,[5,6] with apoptosis of ECs, macrophage adhesion and invasion, and smooth muscle migration and growth, leads to stenosis of the target vessel.[7] Therefore, accelerating re-endothelialization of injured vessels is important for recovery of vascular function, prevention of thrombosis, and inhibition of excessive neointimal hyperplasia.[8,9]

Endothelial regeneration and proliferation after tissue damage, during neoangiogenesis, or after the vascular injury was thought to be a local process managed by the adjacent uninjured ECs within the intact parts of the intima.[10] However, recent studies demonstrated that circulating stem- and bone marrow-derived progenitor cells
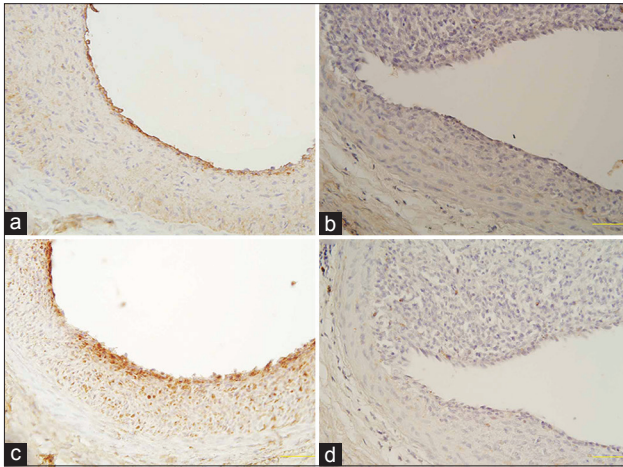
Figure 6: Identification of endothelial progenitor cells in rat common carotid artery (human CD31 and human mitochondria immunohistochemical staining, original magnification, ×400). Human CD31 staining in (a) transplantation group and (b) control group. Human mitochondria staining in (c) transplantation group and (d) control group.

transit through the circulation, enter different organs, and differentiate into cells of the host organ or contribute to the pool of existing stem cells.[11] Recently, with the development of stem cell research, the stem cell transplantation in the treatment of cardiovascular diseases has become a major focus. Stem cell transplantation and its secondary effect of re-endothelialization may become a new method to prevent restenosis after endovascular treatment.

EPCs are mainly isolated from bone marrow, peripheral blood, and umbilical blood by density gradient centrifugation or immune magnetic bead separation method. The EPCs in peripheral blood and umbilical cord blood are also derived from bone marrow. However, use of bone marrow-derived EPCs is limited by the limited number of derived EPCs for its clinical applications,[12] the cells have strong immunogenicity and can only be used for autologous transplantation,[13,14] and background factors in patients including diabetes, hypertension, hypercholesterolemia, aging. Smoking, coronary artery diseases, or other diseases such as emphysema, acute lung injury, liver fibrosis, and systemic sclerosis may decrease the number and biological activity of the cells.[15-21] Thus, how to obtain abundant, high-purity, and humanized EPCs is the primary issue for clinical application.

In our previous study, we isolated EPCs from human early fetal aortas (<14 weeks) and established methods to induce EPC proliferation and differentiation into ECs *in vitro*.[3] Compared with adult tissues, human fetal aortas contain a higher proportion of EPCs and have an active proliferative ability. They also have a low and unique immunogenicity that can be used in xenograft transplantation without the need for immunosupressive agents. The transplanted cells can survive in the host and mediate angiogenesis. In this study, we found that transplanted EPCs could differentiate into ECs in injured arteries, form a fairly complete intima and contribute to re-endothelialization. However, the mechanisms by which

EPCs participate in vascular endothelial repair are not clear so far. EPC-mediated paracrine signaling may play a critical role in re-endothelializion and neovascularization by stimulating proliferation, migration, and survival of ECs.[22,23] Yin *et al*.[21] checked the secretion of cytokines in the culture medium and found significant increase in the expression of vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 during EPC differentiation. Our *in vitro* data also showed that EPCs could differentiate into mature ECs after VEGF treatment (unpublished data).

In conclusion, transplantation of EPCs derived from human early fetal aortas may inhibit neointimal hyperplasia of damaged vessels. The use of humanized EPCs provides an important experimental basis for the clinical application of EPCs after angioplasty and stenting in preventing restenosis of the target vessel. However, further studies on the mechanism of the beneficial effects of the EPC transplantation in promoting vascular repair are still needed. EPC transplantation after endovascular treatment may become an important option for cardiovascular diseases and may improve vascular remodeling and prevent restenosis.

Conflicts of interest
There are no conflicts of interest.

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