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Exosomes derived from endothelial progenitor cells attenuate vascular repair and accelerate reendothelialization by enhancing endothelial function

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Abstract

Background aims. Exosomes, a key component of cell paracrine secretion, can exert protective effects in various disease models. However, application of exosomes in vascular repair and regeneration has rarely been reported. In this study, we tested whether endothelial progenitor cell (EPC)-derived exosomes possessed therapeutic effects in rat models of balloon-induced vascular injury by accelerating reendothelialization. *Methods.* Exosomes were obtained from the conditioned media of EPCs isolated from human umbilical cord blood. Induction of the endothelial injury was performed in the rats' carotid artery, and the pro-re-endothelialization capacity of EPC-derived exosomes was measured. The *in vitro* effects of exosomes on the proliferation and migration of endothelial cells were investigated. *Results.* We found that the EPC-derived exosomes accelerated the re-endothelialization in the early phase after endothelial damage in the rat carotid artery. We also demonstrated that these exosomes enhanced the proliferation and migration of endothelial cells *in vitro*. Moreover, endothelial cells stimulated with these exosomes showed increased expression of angiogenesis-related molecules. *Conclusions.* Taken together, our results indicate that exosomes are an active component of the paracrine secretion of human EPCs and can promote vascular repair in rat models of balloon injury by up-regulating endothelial cells function.

Key Words: *endothelial progenitor cells, exosomes, reendothelialization, vascular repair*

Introduction

Endovascular re-canalization is increasingly being used to restore blood flow to areas of ischemia, tissue loss or gangrene for diabetic patients with peripheral artery disease. However, the early restenosis rate of infrapopliteal occlusions was high, even in lesion treated with drug-eluting balloons [1]. Endothelial cell injury is the central mechanism in the initiation and progression of restenosis. Ongoing deterioration of the endothelial monolayer may induce a continuum of events that give rise to vascular inflammation, lipid deposition, and thrombosis, resulting in neointimal hyperplasia and vessel wall remodeling, which contribute to restenosis [2]. Therefore, an early acceleration of re-endothelialization is essential to prevent progression of restenosis.

Exogenously infused EPCs could regenerate the endothelium by differentiating into mature endothelial cells and by promoting migration and proliferation of existing endothelial cells [3,4]. However, recent studies have revealed that the stimulation of resident endothelial cells from adjacent intact endothelium via paracrine mechanisms might be more important for vascular repair than the direct incorporation and expansion of the exogenously transplanted EPCs during tissue repair [5,6]. Exosomes, small membrane particles (40–100 nm in diameter) originating from multivesicular bodies (MVBs), are an important component of paracrine secretion and play key roles in the intercellular cross-talk via the horizontal transfer of proteins, lipids and RNAs to target cells [7,8]. Recent studies have indicated that the exosomes derived from mesenchymal stromal cells [9] and cardiac

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progenitor cells [10] can trigger angiogenesis and restore tissue function, suggesting a significant role of exosomes in the paracrine action of stem or progenitor cells. Therefore, we hypothesized that the protective effects of EPCs in vascular injuries might be partially mediated by their released exosomes.

In this study, we isolated and purified exosomes released from human umbilical cord blood (UCB)-derived EPCs and determined whether these exosomes could accelerate re-endothelialization in rat models of balloon-induced vascular injury. Moreover, we studied *in vitro* the impact of EPC-secreted exosomes on endothelial cells proliferation, migration and angiogenesis-related genes expression.

Methods

Isolation and culture of EPCs derived from human UCB

Human UCB samples (approximately 80–100 mL per cord) were collected from healthy newborns. This study sought and received informed consent from the parents of the infants and was approved by the Institutional Review Board at Shanghai Six People's Hospital, Shanghai Jiaotong University. Briefly, UCB was diluted 1:1 with phosphate-buffered saline (PBS) and overlaid onto separation medium (Cappel LSM; MP Biomedicals). After centrifugation at 400*g* for 30 min at room temperature, the mononuclear cells (MNCs) layer were collected and washed with PBS. MNCs (5×10^6 cells/cm^2) were then placed into six-well plates pre-coated with type I rat tail collagen (BD Bioscience) and cultured in endothelial growth medium-2 (EGM-2MV; Lonza) containing 5% fetal bovine serum (FBS) and cytokines. Cells were maintained at 37°C and 5% CO_2 in a humidified environment. After 48 h, non-adherent cells were removed with PBS and adherent cells were continuously cultured. The medium was changed daily for 7 days and then every other day until the first passage. EPC colonies were enumerated using an inverted microscope (Leica). Once confluent, EPCs were trypsinized and resuspended in complete EGM-2MV media and plated onto type I collagen pre-coated 25-cm^2 cell culture flasks. Cells were passaged after becoming 80–90% confluent. Early-passage EPCs (p2–6) were used in the subsequent experiments.

Characterization of human UCB-derived EPCs

For immunocytochemistry, the cells were washed with PBS containing 0.5% bovine serum albumin (BSA) and fixed with 4% paraformaldehyde for 15 min. The cells were then permeabilized with 0.1% Triton X-100 for 15 min, blocked with 3% BSA in PBS for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at 37°C. Isotype-irrelevant antibodies were used as negative controls. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL; Invitrogen) for 5 min. The cells were then washed three times and viewed using a fluorescence microscope (Leica). The antibodies, including mouse anti-human CD31, rabbit anti-human von Willebrand factor, and respective secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or Alexa Fluor 488, were obtained from Abcam.

For flow cytometry, 5×10^5 cells were fixed with 4% paraformaldehyde for 15 min, blocked with 3% BSA for 30 min, and then incubated with the following primary antibodies: phycoerythrin (PE)-conjugated mouse anti-human CD133 (MACS technology, Miltenyi Biotec). FITC-conjugated mouse anti-human CD31, allophycocyanin (APC)-conjugated mouse anti-human CD34, PE-conjugated mouse anti-human vascular endothelial growth factor receptor-2 (VEGFR-2), FITC-conjugated mouse anti-human vascular endothelial cadherin (VE-cadherin), and FITC-conjugated mouse anti-human CD45 were obtained from BD Biosciences. Non-specific fluorescence was determined by incubation of similar cell aliquots with isotype-matched mouse monoclonal antibodies. The cells were washed with PBS and analyzed by using the Guava easyCyte Flow Cytometer (Millipore).

Tube formation assay

In vitro capillary-like structure formation was evaluated on growth factor reduced Matrigel (BD Biosciences). Briefly, Matrigel (50 μL/well) was added to 96-well plates and incubated at 37°C for 30 min. The cells (2×10^4 cells/well) were seeded onto the plated Matrigel and cultured at 37°C with 5% CO_2. Tube formation was quantified at 6 h using an inverted microscope.

Assessment of acetylated low-density lipoprotein uptake and Ulex europaeus agglutinin-1 binding

Fluorescent staining was used to detect the uptake of 1,1′-dioctadecyl-3,3,3′,3′- tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (ac-LDL; Dil-ac-LDL, Molecular Probes) and binding of FITC-conjugated *Ulex europaeus agglutinin-1* (UEA-1; FITC-UEA-l, Sigma-Aldrich). Briefly, the cells were incubated with Dil-ac-LDL (15 μg/mL) for 4 h at 37°C and then fixed with 4% paraformaldehyde for 30 min. After washing, the cells were stained with FITC-UEA-l (10 μg/mL) for 1 h at 37°C and with DAPI for 5 min. The cells were washed three times and analyzed under a fluorescence microscope.