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Opinion paper

Endothelial progenitor cells improve functional recovery in focal cerebral ischemia of rat by promoting angiogenesis via VEGF

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ABSTRACT

To investigate the role of venous infusion of endothelial progenitor cells (EPCs) in the reendothelialization of acute focal cerebral ischemia model in rats. And explore the mechanism of VEGF to promote angiogenesis of functional recovery in focal cerebral ischemia of rat. A model of middle cerebral artery occlusion (MCAo) was used to mimic ischemia following EPCs extraction from the same donor rats. EPCs were characterized by CD34, CD45 and CD133 expressions, and confirmed by uptake of fluorescently labeled Dil-ac-LDL and FITC-UEA-1 and flow cytometry analysis. EPCs were expanded in vitro and injected into the jugular vein of the same donor animals daily for 5 days after ischemia surgery. EPC-treated animals received approximately 1×10^6 cells, while control animals received PBS. Animals were evaluated the functional recovery, endothelial cell proliferation, vascular distribution, and VEGF levels. The EPC-treated group showed lower infarct volume and a significant recovery of neurological function. We also observed increased vascular distribution through bromodeoxyuridine (BrdU) staining and high plasma VEGF levels in the EPC-treated group compared to control groups. Our results provided direct evidence that auto-graft EPCs can improve neurological outcome and revascularization after ischemic stroke and indicated an important role of VEGF in this process. Our study suggested that EPCs may have potential therapeutic applications for the ischemic cerebrovascular disease.

1. Introduction

Cerebral ischemia is a condition in which there is insufficient blood flow to the brain to meet metabolic demand. Cerebral ischemia causes alterations in brain metabolism, reduction in metabolic rates, and energy crisis [1]. Cerebral ischemia may lead to many serious symptoms, such as unconsciousness, blindness, problems with coordination, weakness in the body, stroke, cardiorespiratory arrest, and irreversible brain damage. Cerebral ischemia can be divided into focal ischemia and global ischemia, and focal cerebral ischemia is one of the major complications of acute brain injury. Patients with acute brain injury exhibit impaired microvascular endothelial function measured as a decreased circulating level of endothelial progenitor cells (EPCs) [2]. Since Zhang and his colleagues first discovered that EPCs play an important role in ischemic disease [2–4], several studies have shown that EPCs can significantly improve the blood supply of ischemic cerebral areas and effectively promote the recovery of neurological function [5,6].

As we all known, vascular endothelial growth factor (VEGF) is important for almost every aspect of blood vessel formation and function [7]. Numerous studies showed that VEGF expression is indeed a critical determinant of blood vessel development [8,9]. EPCs sprouting migration that leads to the expansion and patterning of the vessel network via angiogenesis, but the underlying mechanism was still unclear. Herein, the relationship between EPCs transplantation and the neurological recovery in rat models needs to be clarified.

2. Materials and methods

All procedures were performed according to an institutionally approved protocol in accordance with Laboratory Animal regulation of China (2001). Sixty Male Sprague-Dawley (SD) rats, 8–12 weeks old and weighing 200–250 g (normal rats), were purchased from Wuhan University animal testing center for use in this study (Permit No: SUXK (E) 2003–2004).

3. Preparation of bone marrow mononuclear cells

Rats were anesthetized and bone marrow cells (3 ml) were extracted from the distal femur under sterile conditions using a 10 ml syringe and stored in heparinized phosphate buffered saline.

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Mononuclear cells (MNCs) were isolated using a Ficoll gradient (Lympholite-M, Cedar lane) [10,11]; (Ficoll-Hypaque, LTS1 083; TBD science, Tianjin, China). The harvest cells were supplemented with M199 media (Sigma-Aldrich) containing 10 ng/ml vascular endothelial growth factor (VEGF, PeproTech Inc, USA), 1 ng/ml basic fibroblast growth factor (b-FGF, PeproTech Inc., USA), 1 ng/ml epidermal growth factor (EGF, PeproTech Inc., USA), 20% fetal bovine serum (FBS, Invitrogen), heparin, penicillin and streptomycin. Cells were incubated, undisturbed, at 37 °C with 5% CO_2 for 3 days. Adherent cell culture continued for two weeks, and media was replaced every 3 days. 1×10^6 cells were re-suspended in PBS for intravenous injection.

4. Animal model and group preparation and treatment

Two weeks after cells extraction, rats were prepared for MCAo. All surgical procedures were approved in accordance with the People's Republic of China on the Ethical Treatment of Animals (2006-9-30). Focal cerebral ischemia was induced as described previously with some modifications [12]. In the sham group only the carotid artery and external carotid meridians were isolated.

After MCAo, animals were randomly assigned to the EPC-treated group, the sham group, and PBS group (n = 15). On this time, the sham group rats only isolated the jugular vein without interfering. EPC-treated group animals were injected with EPCs (1×10^6/ml/kg) while the sham group were injected with PBS (1 ml/kg) through the jugular vein during the acute ischemic phase [13] (2 h after the surgery) for 5 consecutives. On the 6th day, all the rats were given intraperitoneal BrdU (50 mg/kg, Sigma, USA) for 7 consecutive days [14]. Animals were sacrificed 14 days after ischemia procedure. All experimental rats were housed 3 rats per cage in the same environment. Food intake was limited to 30 g per day per animal [15,16]. Body weight, PaO2, PaCO2, pH, blood glucose level, and rectal temperature were not statistically different between the groups. A total of 54 spontaneously hypertensive rats were used in this study. Forty-five rats received MCAo, 15 rats received sham surgery, and 11 died or were excluded from the experiment.

5. Cell immunophenotyping by Dil-Ac-LDL internalization and FITC-UEA-I binding

Immunocytochemistry was performed according to standard protocols [7]and the manufacturer's instructions. Briefly, EPCs were fixed with 4% PFA for 20 min at 4 °C, washed with 3 times with PBS, then incubated overnight at 4C with primary rabbit polyclonal antibodies against rat CD34, CD45 (BioTech, Beijing, China) and CD133 (Boster, Wuhan, China). Following primary antibody incubation, cells were washed 3 times with PBS and incubated with biotinylated secondary antibody (goat anti against rabbit IgG) and streptavidin biotin peroxidase complex (SABC) according to manufacturer's instructions (SA1022, Boster, Wuhan, China). We developed DAB color (ZSGB-BIO, Beijing, China) and counterstained nuclei with hematoxylin.

To assess Ac-LDL internalization and UEA-I binding, we used Dil labeled Ac-LDL (Dil-Ac-LDL, Biomedical Technologies, Inc, Stoughton, USA) and FITC labeled UEA-I (FITC-UEA-I, Sigma-Aldrich), as previously described [17,18]. Samples were imaged using confocal laser scanning microscopy on a Leica TCS SP2 AOBS MP (Germany). On day 14, we incubated live cells with 10 μg/ml Dil-Ac-LDL in M199 media (without other addition) for 4 h at 37 °C. Cells were fixed these with 4% PFA for 20 min at 4 °C, then incubated with 10 μg/ml FITC-UEA-I in PBS for 1 h at 37 °C. Samples were imaged using contra-laser scanning microscopy (LCS Lite).

6. Detection BrdU markers and the labeling percentage

Indirect labeling method was used to detect the BrdU marker rate of different markers by flow cytometry. The Rabbit anti rat BrdU antibody was used as one antibody, the Goat anti rabbit two anti -FITC was two anti -FITC. The cells were collected by 0.25% (mass concentration) trypsin after the cell culture 14 days, and washed by PBS 2 times, the single standard tube 1, 2, and 3 were added to CD34-PE, CD45-PE, and CD133-PE respectively. The samples were incubated avoiding light 30 min at 4 °C. After washing by PBS for 2 times, the single standard tube 1 and 2 were filtered and tested on the machine. The single standard tube 3 was added to two anti -FITC and 4 cm away from light to incubate 30 min and washed by PBS 2 times. The negative control was unlabeled EPC and blank control was replaced by PBS. A normal control group was set up to observe the cytotoxicity of BrdU.

7. Neurological tests

Neurological tests were performed for 3 consecutive days before MCAo and on the 1st, 3rd and 7th day after treatment using a modified neurological severity score [19] (mNSS). Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18) and included motor, sensory, reflex and balance tests. To determine the severity of injury, 1 score point was awarded for the inability to perform the test or lack of a tested reflex. A higher the score indicated more severe injury.

8. Immunohistochemistry

Immunohistochemistry was used to assess cell migration and VEGF expression in the ischemic brain. Immunohistochemistry was performed according to the manufacturer's instructions. Briefly, 5 μm free floating sections were incubated with primary antibody against BrdU (mouse, 1:400, Sigma, USA) overnight at 4C. Sections were then incubated with biotinylated secondary anti-mouse IgM (1:300, Jackson Immunoresearch) for 1 h at room temperature and then counterstained with DAPI (0.1 Ag/ml, Sigma) for 5 min at room temperature. Each section was imaged in the same cortical ischemic zone 5, and BrdU-positive cells were counted as mean ± SD using HPIAS 2000 software analysis. In each case, the proportion of BrdU-positive cells in the contralateral homologous area was also measured in the same manner.

VEGF immunocytochemistry were performed (n = 6) as above using anti-VEGF (Sigma, USA), and VEGF-positive cells were counted as mean ± SD.

Three-Dimensional Image Acquisition and Vascular Analysis.

On days 1, 7 and 14 after EPC transplantation, we examined neovascularization in ischemic brains by intravenous injection of fluorescent isothiocyanate (FITC) dextran (2×10^6 molecular weight, Sigma; 50 mg/mL), n = 6. 48 h later, vascular diameter and number of branch points were measured in three dimensions using previously developed software.

9. ELISA methods to measure the concentrations of plasma VEGF

On days 7 and 14 after surgery, we collected 3 ml blood (n = 6) using EDTA-blood collection tubes. Plasma levels of VEGF were measured using a commercial ELISA assay (R&D Systems, Minneapolis, MN) and analyzed using software to calculate sample concentration from microplate reader values as described previously [20,21]and according to the manufacturer's instructions.

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