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Pro-angiogenic efficacy of transplanting endothelial progenitor cells for treating hindlimb ischemia in hyperglycemic rabbits

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ABSTRACT

Aims: To evaluate the effectiveness of endothelial progenitor cells (EPCs) therapy in ischemia with or without hyperglycemia.
Methods: Japanese White Rabbits were randomly assigned to three groups, group SH, hyperglycemia with sham therapy (n = 10); group NE, normoglycemia with autologous EPCs transplantation therapy (n = 12); and group HE, hyperglycemia with autologous EPCs transplantation therapy (n = 12). Hyperglycemia was induced by injecting alloxan and sustained for 12 weeks. Hindlimb ischemia was induced by complete excision of the femoral artery. Ex vivo-expanded EPCs were derived from autologous bone marrow and transplanted intermuscularly in the ischemic hindlimb. Fourteen days after transplantation, the indicators were determined.
Results: There is no difference of the functions of ex vivo-expanded EPCs from autologous bone marrow between normoglycemic and hyperglycemic groups. We found significant improvement in both EPCs transplantation therapy groups compared to sham, in terms of the angiogenesis index (8.62 ± 1.36, 11.12 ± 2.23, 12.35 ± 2.97), capillary density (7.06 ± 0.91, 13.51 ± 1.16, 13.90 ± 2.78), capillary to muscle fiber ratio (0.68 ± 0.09, 0.96 ± 0.11,0.89 ± 0.10), muscle VEGF expression (0.22 ± 0.07, 0.41 ± 0.08, 0.38 ± 0.07 ng/g). We found no significant differences between hyperglycemic and normoglycemic EPCs therapy groups except for 5 pro-angiogenic genes that were upregulated in HE as compared to NE.
Conclusion: Ex vivo expanded EPCs from autologous bone marrow transplantation is an effective therapeutic method for hindlimb ischemia in rabbits regardless of glycemic state.

1. Introduction

Endothelial progenitor cells (EPCs) are precursors to vascular endothelial cells, the effective components of mononuclear cell assortments used to treat ischemic disease (Bassi et al., 2012; Devanesan, Laughlan, Girn, & Homer-Vanniasinkam, 2009; Liao et al., 2014). Liao et al. (2014) has reported that coronary heart disease risk equivalence in diabetes and arterial diseases is characterized by endothelial function and endothelial progenitor cell. It has been reported that transplantation of ex vivo expanded EPCs induces vascular remodeling and improves circulation (Thal et al., 2012; Vinik, Erbas, & Casellini, 2013). Tal et al. (Faglia et al., 2012) has reported that transplantation of healthy outgrowth endothelial cell (OECs) may rescue the ischemic myocardium by neovasculogenesis and paracrine effect in diabetic rabbits. Therefore, EPCs may offer a novel method for treating ischemic diseases arising from both micro and macrovascular complications associated with long term hyperglycemia (Desouza, 2013; Faglia et al., 2012; Lu et al., 2011; Tan et al., 2010; Yiu & Tse, 2014). However, there is not enough evidence to determine whether hyperglycemia attenuates the efficacy of EPCs therapy in hindlimb ischemia. To address this possibility, we performed ex vivo expansion and transplantation of bone marrow-derived EPCs, using established methodology (Cheng, Hu, Lv, Ling, & Jiang, 2012; Jeon et al., 2007; Zhang et al., 2012), into the ischemic hindlimb of normoglycemic and hyperglycemic rabbits. We then assess key markers of angiogenesis in three experimental groups, hyperglycemic with EPCs therapy (HE), nomoglycemia with EPCs therapy (NE) and

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sham. Our data support the use of EPCs as an effective therapy for hindlimb ischemia as its efficacy is not diminished by hyperglycemic state.

2. Materials and methods

2.1. Animals

Thirty-four Japanese White Rabbits weighing 2.0–2.5 kg and with blood glucose ≤7 mmol/L were randomly allocated into three groups. The first received experimentally induced hyperglycemia and underwent PBS transplantation therapy (group SH, n = 10), or normoglycemia and underwent autologous EPCs transplantation therapy (group NE, n = 12) or experimentally induced hyperglycemia with autologous EPCs transplantation therapy (group HE, n = 12). There was no loss of experimental animal due to trauma, complications, or infections.

2.2. Establishment of hyperglycemia

The rabbits were fasted for 24–48 hours before 3% alloxan (Sigma, St. Louis, MO, USA) was injected into the ear vein (80 mg/kg) over 30 s. Rabbits were considered hyperglycemic if blood glucose was ≥16 mmol/L (caudal vein, ACCU-CHEK Performa glucose meter, ROCHE) at 72 hours after injection; if not, a second dose of alloxan (80 mg/kg) was injected. Blood glucose levels were measured weekly, and additional doses of alloxan were administered as needed to maintain values ≥16 mmol/L. (SDC Table S1) (Kashiwagi, et al., 2012; Seino et al., 2010) The animals were kept in the hyperglycemic state for 12 weeks before harvesting bone marrow.

2.3. Collection and culture of EPCs

The rabbits were anesthetized with 1% pentobarbital sodium through ear vein (30 mg/kg), the left tibia was exposed, and 5 ml of bone marrow was extracted from each rabbit. Bone marrow was then mixed with lymphocyte separating solution and centrifuged at 2000 RPM for 20 min to isolate mononuclear cells. Mononuclear cells were then transferred to a 10-ml aseptic centrifuge tube containing phosphate-buffered saline (PBS), further washed with PBS, and inoculated into a fibronectin-covered culture flask at a density of 5×10^6 cells/cm^2. The cells were cultured at 37 °C, under 5% CO_2 and saturated humidity in M199 (SIGMA product, #M 4530, Liquid, with Earle's salts, L-glutamine, and sodium bicarbonate; sterile-filtered, including 5% FBS) induced culture liquid. Medium was first changed after 4 days, and suspended cells were removed. Medium was further changed every 3 days until cells were transplanted at 7–10 days.

2.4. Staining and identification of EPCs

Cells were placed in culture dishes and immersed in 2% paraformaldehyde for 10 min, following incubation of cells with acLDL-Dii (10 μg/ml) at 37 °C for 24 h. Cells were then washed with PBS and incubated with FITC-UEA-I (10 μg/ml) for 2 h. The EPCs were visualized under laser scanning confocal fluorescence microscope, and adherent cells were identified by FITC-UEA-I staining and acLDL-Dii uptake. At the same time, adherent cells were detected for phycoerythrin (PE)-CD34, PE-CD133 and PE-kinase insert domain receptor (PE-KDR) expression by flow cytometry, controlled by the corresponding PE-IgG1.

2.5. EPCs adhesion and migration

Trypsin was used to digest the adherent EPCs, which were then collected, added to M199 medium (including 5% FBS), counted, then inoculated onto a culture board coated with human fibronectin. The cells were left to culture for 30 min in a 37 °C incubator. We then washed out non-adherent cells with PBS and counted the number of adherent cells. For the detection of EPC migration, we collected adherent EPCs, added them to M199 medium and counted them. Endothelial basal medium (EBM) medium and vascular endothelial growth factor (VEGF,50 μg/ml) were added to the inferior chamber of the modified Boyden chamber, and 2×10^4 EPCs suspended in 50 μl medium were added to the superior chamber. After cultivating for 24 h, non-moving cells on the filter membrane in the superior chamber were removed. All other cells in the inferior chamber were fixed by methanol, stained by Giemsa, then three fields were selected randomly, and cells that had migrated to the underlayer were counted.

2.6. Establishment of hindlimb ischemia and cell transplantation

Femoral arterectomy was performed to establish hindlimb ischemia, as previously described (Hong et al., 2001). Rabbits were then injected with 20 μl of PBS (SH group) or PBS containing 2×10^6 EPCs (NE and HE groups) into muscle of the ischemic hindlimb of the same rabbit the cells were obtained from

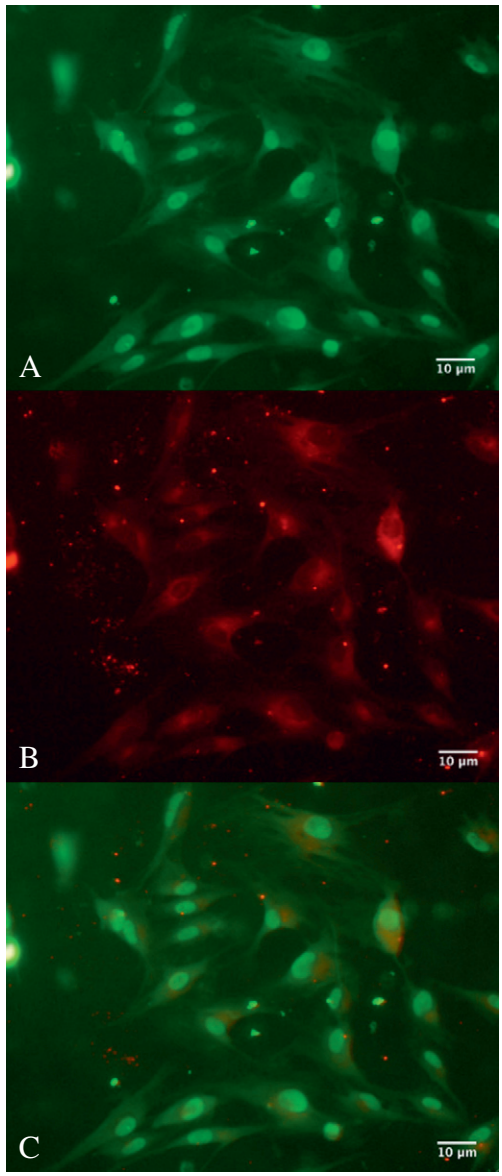

Fig. 1. Characterization of EPCs observed under a laser-scanning confocal microscope (bar = 20um). Staining for lectin (A green, excitation wavelength 543 nm), acLDL-Dii (B red, excitation wavelength 633 nm). Double-staining (C yellow) confirmed the presence of EPCs (A, B, C).