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ADIPOSE DERIVED CELLS

Adipose-derived stem cell spheroid treated with low-level light irradiation accelerates spontaneous angiogenesis in mouse model of hindlimb ischemia

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Abstract

Background aims. We investigated whether low-level light irradiation (LLLI) before adipose-derived stromal cells (ASCs) spheroid transplantation improved hind-limb functional recovery by stimulation of angiogenesis. *Methods.* The spheroid, composed of ASCs, was irradiated with low-level light and expressed angiogenic factors, including vascular endothelial growth factor and basic fibroblast growth factor. From immunochemical staining analysis, the spheroid of ASCs included $CD31^+$, KDR^+ and $CD34^+$, whereas monolayer-cultured ASCs were negative for these markers. To evaluate the therapeutic effect of the ASC spheroid treated with LLLI *in vivo*, phosphate-buffered saline, monolayer ASCs, LLLI-monolayer ASCs, spheroid ASCs and LLLI-spheroid ASCs were transplanted into a hind-limb ischemia model. *Results.* The LLLI-spheroid ASCs transplanted into the hind-limb ischemia differentiated into endothelial cells and remained differentiated. Transplantation of LLLI-spheroid ASCs into the hind-limb ischemia significantly elevated the density of vascular formations through angiogenic factors released by the ASCs and enhanced tissue regeneration at the lesion site. Consistent with these results, the transplantation of LLLI-spheroid ASCs significantly improved functional recovery compared with ASC or spheroid ASC transplantation and PBS treatment. *Conclusions.* These findings suggest that transplantation of ASC spheroid treated with LLLI may be an effective stem cell therapy for the treatment of hind-limb ischemia and peripheral vascular disease.

Key Words: *adipose-derived stromal cell, angiogenesis, endothelial differentiation, low-level light irradiation, spheroid*

Introduction

Angiogenesis is required for healing of ischemic limbs. Angiogenesis aids in damaged tissue repair by regenerating blood vessels and thus improving blood flow after limb ischemia [1]. Stem cell therapy is a relatively new field in the battle against ischemic vascular disease that has sparked intense research and criticism [2]. Endothelial cell lineage stem cell–derived endothelial progenitor cells and stem cells were previously examined for therapeutic angiogenesis in the treatment of cardiac infarction and ischemia [3]. Primarily single cells were injected into the target site. However, many limitations remain regarding the use of stem cells in clinical applications, including low therapeutic efficiency and poor cell survival, because the cells themselves lack well-developed vascular tissues. Cells transplanted into ischemic regions are exposed to low oxygen conditions after transplantation due to the lack of blood supply and are prone to apoptosis [4]. Cell-cell contact is the minimum requirement to render cells responsive to the activities of survival factors [5]. Correspondingly, three-dimensional (3D) cell aggregates prevent cell apoptosis and promote stabilization. It has been reported that hypoxic preconditioning results in enhanced survival of human mesenchymal stromal cells [6]. Because cells within the spheroid are naturally exposed to mild hypoxia, they are preconditioned to an ischemic environment. Spheroids of stem cells have improved

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therapeutic efficacy via enhanced cell viability and paracrine effects in ischemia models. Hypoxia stimulates the production of growth factors such as vascular endothelial growth factor (VEGF), which induce angiogenesis and endothelial cell survival. In two-dimensional (2D) cultures, growth factors secreted from cells are released and diluted in the culture supernatant, preventing cells from responding to the released factors. Several experimental strategies for functional endothelial differentiation of stem cells have been developed, including 2D cell culture in endothelial cell growth medium containing VEGF and fibroblast growth factor (FGF), 3D spheroid culture on substrates with immobilized polypeptides, and genetic modification of stem cells [6–8]. However, there are no reports on high-ratio endothelial cell differentiation of adipose-derived stromal cells (ASCs) in 3D-cultured stem cells without growth factors and peptides. Genetic modification of stem cells has been hindered by the lack of safe and effective gene delivery vehicles because even highly efficient viral vectors possess safety concerns such as immunogenicity, mutagenesis or potential toxicity [6]. In addition, the use of nonviral vectors suffers from low delivery efficiency. It has been reported that low-level light irradiation (LLLI) improves cell migration at low levels of red light illumination [9]. The proliferation and growth factor secretion of ASCs were also enhanced by LLLI [9]. Moreover, red and infrared light-emitting diode (LED) enhanced tissue healing by stimulating angiogenesis in various animal models of ischemia [10].

In this study, LLLI was used to promote what we refer to as a hypoxic ASC spheroid and stimulate cellular migration by weakening cell-matrix adhesion. Proliferation and growth factor (FGF and VEGF) secretion were also enhanced by LLLI. ASCs can differentiate into endothelial cells without endothelial cell growth medium containing VEGF and FGF. Vasculogenic activity and potential therapeutic efficacy of human ASC spheroid treated with LLLI was evaluated by injecting ASC spheroid with LLLI (LLLI-spheroid) into a mouse hind-limb ischemic model.

Methods

Low-level light irradiation

LED (WON Technology) was applied for 10 min daily from day 1 to 3. The LED was designed to fit over a standard multi-well plate (12.5 × 8.5 cm) for cell culture and had an emission wavelength that peaked at 660 nm. The irradiance at the surface of the cell monolayer was measured by a power meter (Orion, Ophir Optronics). To obtain the energy dose of 6 J/cm^2, exposure time for LED array was 10 min under a power density of 10 mW/cm^2 (1 milliwatt × second = 0.001 joules).

ASC culture

The ASCs were supplied by Cell Engineering for Origin under a material transfer agreement. ASCs were isolated from adipose tissue and cultured in low-glucose Dulbecco's modified Eagle's medium F-12 (Welgene) supplemented with 10% fetal bovine serum (Welgene), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator. The ASCs between passages 5 and 8 were used for all experiments. Additionally, the LLLI group received LED treatment.

Spheroid formation

ASCs were split and seeded on a 24-well polystyrene plate (low cell binding surface) at a density of 7.5×10^4 cells/cm^2 (15×10^4 cells/well) and allowed to adhere at 37°C in a 5% CO_2 incubator. Within 3 days of culture, ASCs formed spheroids. Spheroid sizes were measured by counting the area of individual cell clusters using image analysis. The diameters of spheroids were presented as median ± SD (n = 8 per group).

Fluorescence-activated cell sorting

The cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and stained in PBS containing 1% BSA, with either isotype controls or antigen-specific antibodies, for 60 min. CD34 (BD Biosciences), KDR (Beckman Coulter), CD31 (Beckman Coulter), CD45 (Abcam), CD90 (BD Biosciences), CD105 (Caltac Laboratories) and CD29 (Millipore) human antibodies were used. The cells were washed three times with PBS containing 0.5% BSA and were resuspended in PBS for flow cytometry using an Accuri device (BD Biosciences). The isotype immunoglobulin (Ig)G was used as a negative control.

Enzyme-linked immunosorbent assay for angiogenic growth factor production

Angiogenic growth factor production in the LLLI-spheroid was assayed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocols. Concentrations are expressed as the amount of angiogenic growth factor per 10^4 cells at a given time.

Immunofluorescence staining

Indirect immunofluorescence staining was performed using a standard procedure. In brief, tissues cryosectioned into 4-μm-thick sections were fixed with 4% paraformaldehyde, blocked with 5% BSA/PBS (1 h, 24°C), washed twice with PBS, treated with 0.1% Triton X-100/PBS for 1 min and washed extensively in PBS. The sections were stained with specific primary anti-