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Enhanced angiogenic effect of adipose-derived stromal cell spheroid with low-level light therapy in hind limb ischemia mice

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ABSTRACT

The aim of this study was to investigate the effects of low-level laser therapy (LLLT) on transplanted human adipose-derived mesenchymal stem cells (hASCs) spheroid in a hind limb ischemia animal model. LLLT, hASCs spheroid and hASCs spheroid transplantation with LLLT (spheroid + LLLT) were applied to the ischemic hind limbs in athymic mice. The survival, differentiation and secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) of the spheroid ASCs were evaluated by immunohistochemistry and western blots. Spheroid + LLLT group had enhanced the tissue regeneration, including angiogenesis, compared with the ASC group. The spheroid ASCs contributed to tissue regeneration *via* differentiation and secretion of growth factors. In the spheroid + LLLT group, the survival of spheroid hASCs increased with a concomitant decrease in apoptosis of spheroid hASCs in the ischemic hind limb. The secretion of growth factors was stimulated in the spheroid + LLLT group compared with the ASCs and spheroid group. These data suggested that LLLT is an effective biostimulator of spheroid hASCs in tissue regeneration that enhanced the survival of ASCs and stimulated the secretion of growth factors in the ischemic hind limb.

1. Introduction

Critical limb ischemia (CLI) is the most severe form of peripheral arterial disease caused by chronic inflammatory processes associated with atherosclerosis that result in markedly reduced blood flow to the legs, feet and hands [1]. The only current clinical treatment for CLI is endovascular or surgical revascularization. Angiogenesis is required for healing of ischemic limbs. Angiogenesis aids in damaged tissue repair by regenerating blood vessels and thus improves blood flow and oxygen supply after limb ischemia [2]. Recent stem cell therapy clinical trials have aimed at increasing vascularization sufficient for wound perfusion and healing to occur. Such therapy has promising results at preventing limb amputation [3]. Mesenchymal stem cells (MSC) isolated from either bone marrow or adipose tissue induced angiogenesis mainly *via* paracrine secretion of angiogenic growth factors [4]. Unfortunately, limitations still remain regarding the clinical application of stem cells, predominately because of their low therapeutic efficacy [5,6]. Most of the applied stem cells die within 1 week of transplant. Moreover, the transplanted stem cells do not undergo angiogenesis themselves, rather paracrine factors released by the transplanted cells are actually responsible for enhancing host angiogenesis [7].

Several experimental strategies for improving the survival and engraftment of stem cells in ischemic tissue have been developed, including transplantation in combination with growth factor delivery, genetic modification of stem cells, and cell spheroid culture [8–10]. Although transplantation in combination with growth factor delivery improved the survival of stem cells, this strategy still has problems for wider clinical application [9]. Genetic modification of stem cells has been hindered due to the lack of safe and effective gene delivery vehicles. Three-dimensional cell spheroids prevent cell apoptosis and promote cell stabilization after engraftment in ischemic tissue [8]. Grafting stem cell as spheroids has improved therapeutic efficacy *via* enhanced cell viability and paracrine effects in ischemia models [9]. Since cells within the spheroid are naturally exposed to mild hypoxia they are naturally

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preconditioned to an ischemic environment. Therefore, spheroid grafting in ischemic tissues may render cells resistant to hypoxia compared to dissociative grafting. However, these strategies require large initial numbers of cells.

It is well known that the proliferation and growth factor secretion of hASCs was also enhanced by low-level light therapy (LLLT) [11]. Moreover, the use of LLLT to stimulate new blood vessel growth has recently received considerable attention [12]. Red and infrared light emitting diode (LED (660 nm)) enhanced tissue healing by stimulating angiogenesis in various animal models of ischemia [13]. However, little is known about the effects of LLLT on transplanted stem cells in animal models of hind limb ischemia.

This study investigated whether spheroid delivery of hASCs with LLLT enhanced therapeutic efficacy in a mouse model of hind limb ischemia when compared to delivery of dissociated hASCs. First, hASCs were cultured as spheroids, and expression of hypoxia-induced survival factors, inhibition of apoptosis, and extracellular matrix (ECM) preservation were examined. Next, hASCs were grafted to mouse ischemic hind limbs as spheroids, spheroids with LLLT or dissociated cells, and survival, angiogenic factor secretion, and angiogenic efficacy of the grafted hASCs were examined.

2. Materials and methods

2.1. Culture of ASCs

hASCs supplied from CEFO (Seoul, Korea) were cultured in low-glucose Dulbecco's modified Eagle's medium F-12 (DMEM/F-12; Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene), 100units/ml penicillin and 100 μg/ml streptomycin at 37.0 °C in a 5% CO_2 incubator. hASCs between passage 5 and 8 were used for all experiments.

2.2. Spheroid formation

hASCs were split and seeded on adhesion substrates in Dulbecco's modified Eagle's medium F-12 (DMEM F-12; Welgene, Daegu, Korea) with 10% FBS (Welgene) at a density of 7.5×10^4 cells/cm^2, and allowed to adhere at 37 °C. Within 3 days of culture on non-tissue culture-treated 24-well plates, hASCs formed spheroids. Spheroid sizes were measured by counting the area of individual cell clusters by image analysis. The diameters of Spheroids were presented as median ± SD ($n = 10$ per group).

2.3. Cells viability assay

The cell viability of spheroids was analyzed by using a live/dead viability cytotoxicity assay kit after 3 day in culture (Molecular probes, Carlsbad, CA). Briefly, 1 ml of HEPES-buffered saline solution (HBSS) containing 2 μl of green (SYTO 10 fluorescent nucleic acid stain solution) and 2 μl of red (ethidium homodimer-2) nucleic acid stain solution was added to plates and then incubated at 37 °C in a 5% CO_2 incubator for 15 min. The negative control was prepared by freezing cells at −80 °C for 30 min. Images were quantified by using ImageJ software (NIH, Bethesda, MD). The percentage of live/dead cells was scored by counting number of pixels per image.

2.4. Fluorescence-activated cell sorting (FACS)

Cells were washed with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO). The cells were stained in PBS containing 1% BSA with either isotype controls or antigen specific antibodies for 60 min. The antibodies used were human CD34 (BD Biosciences, San Jose, CA), KDR (Beckman Coulter, Brea, CA), CD31 (Beckman Coulter), CD45 (Abcam, Cambridge, MA), CD90 (BD Biosciences), CD105 (Caltac Laboratories, Burlingham, CA) and CD29 (Millipore, Waltham, MA). The cells were washed thrice with PBS containing 0.5% BSA and resuspended in PBS for flow cytometry using an Accuri device (BD Biosciences). Isotype control IgG was used as a negative control.

2.5. Human angiogenic protein analysis

For analyzing the expression profiles of angiogenesis-related proteins, we used the Human Angiogenesis Array Kit (R&D Systems, Ltd., Abingdon, UK). Cell samples (5×10^6 cells) were harvested and 150 μg of protein was mixed with 15 μl of biotinylated detection antibodies. After pre-treatment the cocktail was incubated with the array overnight at 4 °C on a rocking platform. Following a wash step to remove unbound material, streptavidin–horseradish and chemiluminescent detection reagents are added sequentially. The signals on membrane film were detected by scanning on an image reader LAS-3000 (Kodak, Rochester, NY) and quantified using MultiGauge 4.0 software (Kodak). The positive signals seen on developed film can be identified by placing the transparency overlay on the array image and aligning it with the two pairs of positive control spots in the corners of each array.

2.6. ELISA assay for angiogenic growth factor production

Angiogenic growth factor production in the spheroid was assayed with a commercially available 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.

2.7. Histological staining

Ischemic limb muscles were harvested 21 days post-treatment. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated in a graded ethanol series and embedded in paraffin. Specimens were sliced into 4 μm-thick sections and stained with Hematoxylin and Eosin (H&E) to examine muscle degeneration and tissue inflammation. Masson's trichrome collagen staining was performed to assess tissue fibrosis in ischemic regions.

2.8. 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 antibodies and fluorescent-conjugated secondary antibodies (Supplementary Table 1) using a M.O.M kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). The cells were counterstained with DAPI (4,6-diamino-2-phenylindole dihydrochloride; Vector Laboratories). Negative control-mouse IgG (Dako, Carpinteria, CA) and −rabbit IgG (Dako) antibody were used as negative controls. To detect transplanted human cells, sections were stained by immunofluorescence with anti-human nuclear antigen (HNA, Millipore). Stained sections were viewed with a model DXM1200F fluorescence microscope (Nikon, Tokyo, Japan). Processed images were analyzed for fluorescence intensity using ImageJ software (NIH, Bethesda, MD).

2.9. Western blot analysis

Samples were solubilized in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin) for 1 h at 4 °C. Lysates were clarified by centrifugation at 15,000 g for 30 min at 4 °C, diluted in Laemmli sample buffer containing 2% SDS and 5% (v/v) 2-mercaptoethanol, and heated for 5 min at 90 °C. Proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) using 10% or 15% resolving gels followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody for 1 h at room temperature. For detection, peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) were used as described by the manufacturer. Membranes were scanned to create chemiluminescent images and quantified with an image analyzer (Kodak).

2.10. Preparation of the experimental animal model

The animal studies were approved by the Dankook University Animal Use and Care Committee. Five-week-old male BALB/c nude mice (20 g body weight; Narabio, Seoul, Korea) were anesthetized with Zoletil (30 mg/kg). The femoral artery and its branches were ligated through a skin incision using a 5-0 silk suture. The external iliac artery and all of the above arteries were then ligated [4,9]. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries.

2.11. Treatment of limb ischemia

One day after arterial dissection, the mice were divided randomly into 3 experimental groups. hASCs (15×10^5 cells; hASCs group, $n = 9$) cultured on TCP for 3 days; or spheroid (10 masses; spheroids group, $n = 9$) cultured on non–tissue culture–treated 24-well plates for 3 days were mixed with PBS (300 μl) and injected intramuscularly into 3 sites of the gracilis muscle in the medial thigh of the ischemic limb. An equivalent number of cells were injected in both conditions. The control group received an injection of PBS (PBS group, $n = 9$). Physiological status of ischemic limbs was followed-up until 21 days post-treatment. After 3 weeks, cutaneous blood flow was measured by a Laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System PeriScan PIM 3 System; Perimed AB, Stockholm, Sweden). Mice were placed on a heating plate at 37 °C before scanning was initiated. After laser Doppler color image had been recorded twice, the average perfusion in ischemic and non-ischemic limbs was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature, perfusion is expressed as the ratio of the ischemic to non-ischemic hind limb. The outcome was rated in 3 levels: limb salvage (similar limb integrity and morphology as the normal limb control of the same animal), foot necrosis (death of the foot tissue), or hind limb loss.