Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Adipose-derived stromal cell cluster with light therapy enhance angiogenesis and skin wound healing in mice

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ARTICLE INFO

Article history:
Received 7 April 2015
Available online xxx

Keywords:
Wound healing
Skin regeneration
Adipose-derived mesenchymal stem cells
Low-level light therapy
Cluster culture

ABSTRACT

Human adipose-derived mesenchymal stem cells (hASCs) are attractive cell source for skin tissue engineering. The aim of this study was to investigate the effects of low-level light therapy (LLLT) on transplanted cluster hASC in a skin wound animal model. The hASCs were cultured in monolayer or clusters. The LLLT, hASCs, hASC clusters, and hASC clusters transplantation with LLLT (cluster + LLLT) were applied to the wound bed in athymic mice. Wound healing was assessed by gross evaluation and by hematoxylin and eosin staining, and elastin van gieson histochemistry. The survival, differentiation, and secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), and hepatocyte growth factor (HGF) of the cluster ASC were evaluated by immunohistochemistry and Western blotting. The cluster + LLLT group enhanced the wound healing, including neovascularization and regeneration of skin appendages, compared with the cluster group. The secretion of growth factors was stimulated in the cluster + LLLT group compared with the ASCs and cluster group. These data suggest that LLLT is an effective biostimulator of cluster hASCs in wound healing that enhances the survival of hASCs and stimulates the secretion of growth factors in the wound bed.

1. Introduction

Healing of a wound is a complex chain of events with interactions among different cells and tissues, and protracted process of tissue repair [1]. Although various solutions have been recommended for cleansing wounds, the treatment of many chronic wounds remains unsatisfactory and more effective treatment strategies are needed. The ultimate goal for tissue engineers is to regenerate skin such that the complete structural and functional properties of the wounded area are restored to the levels before injury [1]. Angiogenesis, the formation of new blood vessels, is necessary for wound repair since the new vessels provide nutrients to support the active cells [2]. Recent stem cell therapy clinical trials aimed at increasing vascularization to be sufficient for wound perfusion and healing [3].

Human adipose-derived mesenchymal stem cells (hASCs), which are found in adipose tissue, are an attractive cell therapy source for the regeneration of damaged tissues because they are the ability of self-renewal and the ability to differentiate into various cell lineages [4,5]. Transplanting hASCs induces neovascularization and improves blood flow to ischemic tissue in animal models [6,7]. In spite of the angiogenic potential of hASCs for treatment of ischemic wounds, these cell sources have limitations for therapeutic angiogenesis. Although ASCs are favorable with regard to obtaining the number of cells required for transplantation, few transplanted stem cells have been found to differentiate into endothelial cells (ECs) and incorporate into vascular structures in ischemic sites [8]. Most of the applied stem cells die within 1 week of transplantation [9]. Several strategies for improving the survival and engraftment of stem cells in ischemic tissue have been developed [10–12]. In recent studies, it has been reported that hypoxic preconditioning results in improved therapeutic potential of human mesenchymal stem cells. Since cells at the cluster are naturally exposed to mild hypoxia, these cells are naturally preconditioned to an ischemic environment [13]. Therefore, cluster grafting to ischemic tissues may render cells to be resistant to hypoxia.

Low-level light therapy (LLLT) has been used for a long time for various purposes, such as reduce inflammation and improvement

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http://dx.doi.org/10.1016/j.bbrc.2015.04.059
0006-291X/

in the local circulation. Moreover, many studies have demonstrated positive bio-stimulatory effects of LLLT on stem cells [14]. Red Light emitting diode (660 nm) also enhanced tissue healing by stimulating angiogenesis in various animal models of ischemia [15].

This study was performed to determine the effect of LLLT on transplanted cluster hASC in a skin wound animal model. The hASCs were grafted to wound beds of athymic mice as clusters and clusters with LLLT or dissociated cells, and the survival, angiogenic factor secretion, and angiogenic efficacy of the grafted hASCs were examined.

2. Materials and methods

2.1. Culture of ASCs

The 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), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37.0 °C in a 5% CO_2 incubator. The hASCs between passage 5 and 8 were used for all experiments.

2.2. Cluster formation

hASCs were split and seeded on 24 well polystyrene plate (low cell binding surface) at a density of 7.5×10^4 cells/cm^2, and allowed to adhere at 37 °C. Within 3 days of culture, hASCs formed clusters [10]. Cluster sizes were measured by counting the area of individual cell clusters by image analysis. The diameters of clusters were presented as median ± SD (n = 10 per group).

2.3. 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. 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 were 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.

2.4. ELISA assay for angiogenic growth factor production

Angiogenic growth factor production in the cluster 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.

2.5. Histological staining

Samples were harvested 14 days after treatment. 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 fibrosisin ischemic regions. The criteria used for the histological scores of wound healing were modified from previous reports [16] and are summarized in Supplemental Table 2. The histological parameters were reepithelialization, dermal regeneration, granulation tissue formation, and angiogenesis. The regeneration of skin appendages was assessed by counting the number of hair follicles or sebaceous glands in the wound bed.

2.6. Immunofluorescence staining

Indirect immunofluorescence staining was performed using a standard procedure. In brief, tissues cryosectioned at 4 μm thickness 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 (Supplemental 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 was used as a negative control. The stained sections were viewed with a fluorescence microscope of model DXM1200F (Nikon, Tokyo, Japan). Processed images were analyzed for fluorescence intensity using ImageJ software (NIH).

2.7. Western blot analysis

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). 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 to quantify with an image analyzer (Kodak).

2.8. 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 ketamine (100 mg/kg). After aseptically preparing the surgical site, two-full thickness skin wounds were created on the dorsal part using a 8 mm biopsy punch. To inhibit wound contraction, 0.5 mm thickness of silicone splint was applied as described previously [17]. The wounds were randomly classified into five groups: control ($n = 9$), LLLT ($n = 9$), ASCs (15×10^5 cells; hASCs group, $n = 9$), cluster (10 masses; clusters group, $n = 9$), and cluster + LLLT (10 masses; clusters group, $n = 9$). In the ASCs, cluster, and cluster + LLLT groups, 15×10^5 cells ASCs in 100 μl of PBS were transplanted intradermally at four injection sites on the border between the wound and the normal skin. The control group received an injection of PBS (PBS group, $n = 9$). The physiological status of ischemic limbs was followed up to 2 weeks after treatment. Tegaderm (3M Health Care, MN, USA) was used for wound protection. An equivalent number of cells were injected in both conditions.

2.9. Low-level light therapy

Light emitting diode (LED; WON Technology, Daejeon, Korea) was applied for 10 min daily from day 1–13. The distance from the LED to the wound was 8 cm. This LED model exhibited an irradiated wavelength of 660 nm and power density of 50 mW/cm^2. The fluence of each wound site was approximately 30 J/cm^2 (1 mW × second = 0.001 J).

2.10. Gross evaluation of the wound area

The wounds were photographed using a digital camera at 3, 7, and 14 days after surgery. The wound area was measured by tracing the wound margin and calculated using an image analysis program (Image J, NIH, MD, USA). The wound area was analyzed as a

Please cite this article in press as: I.-S. Park, et al., Adipose-derived stromal cell cluster with light therapy enhance angiogenesis and skin wound healing in mice, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.04.059