Cytotherapy, 2015; 17: 283–292

International Society for Cellular Therapy
ISCT

Prevention of skin flap necrosis by use of adipose-derived stromal cells with light-emitting diode phototherapy

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Abstract

Background aims. The aim of this study was to investigate the effects of low-level light therapy (LLLT) on transplanted human adipose-derived mesenchymal stromal cells (ASCs) in the skin flap of mice. *Methods.* LLLT, ASC transplantation and ASC transplantation with LLLT (ASC + LLLT) were applied to the skin flap. Immunostaining and Western blot analysis were performed to evaluate cell survival and differentiation and secretion of vascular endothelial growth factor and basic fibroblast growth factor by the ASCs. Vascular regeneration was assessed by means of immunostaining in addition to hematoxylin and eosin staining. In the ASC + LLLT group, the survival of ASCs was increased as the result of the decreased apoptosis of ASCs. *Results.* The secretion of growth factors was higher in this group as compared with ASCs alone. ASCs contributed to tissue regeneration through vascular cell differentiation and secretion of angiogenic growth factors. The ASC + LLLT group displayed improved treatment efficacy including neovascularization and tissue regeneration compared with ASCs alone. Transplanting ASCs to ischemic skin flaps improved therapeutic efficacy for ischemia treatment as the result of enhanced cell survival and paracrine effects. *Conclusions.* These data suggest that LLLT is an effective biostimulator of ASCs in vascular regeneration, which enhances the survival of ASCs and stimulates the secretion of growth factors in skin flaps.

Key Words: *angiogenesis, ASC, low-level light therapy, skin flap survival, VEGF*

Introduction

Flap surgery is commonly used to reconstruct large areas of skin, damaged after accidental trauma or surgical procedures, as in cancer excisions [1]. However, necrosis represents a major complication that may require secondary surgical interventions and delay future treatments. Necrosis is caused by severe ischemia, resulting from impaired arterial inflow, especially in the distal part of the flap [1]. The goal of therapeutic angiogenesis is to treat ischemic skin flaps by stimulating new blood vessel growth from pre-existing vessels [2]. Additionally, the administration of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) promote angiogenesis; however, the prevention of flap necrosis is compromised by a short half-life of the agents and potential side effects [3]. However, the safety of this approach remains controversial, and no efficient therapy is currently available.

Recently, stem cells have drawn great attention in the treatment of ischemic flaps [1,4]. Adipose-derived mesenchymal stromal cells (ASCs), which are found in many adult tissues, are an attractive cell therapy source for the regeneration of damaged tissues because they are able to self-renew and are capable of differentiating into various cells and tissues [5,6]. Transplanting human ASCs (hASCs) induces neovascularization and improves blood flow to ischemic tissue in animal models [7,8]. It has also been demonstrated that growth factors and cytokines released by ASCs promote *in vitro* and *in vivo* arteriogenesis in ischemic tissue through paracrine mechanisms [7,8]. Thus, ASCs could be a novel source of cell therapy for ischemic tissues. Unfortunately, several studies have reported that stem cell therapy has minimal effects. Most of the applied stem cells die within 1 week of transplantation. Therefore, to develop successful stem cell therapies, it is necessary to cultivate stem cells that can survive in

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(Received 16 July 2014; accepted 23 October 2014)

ISSN 1465-3249 Copyright © 2015, International Society for Cellular Therapy. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jcyt.2014.10.017

ischemic tissue while being capable of differentiation into vascular cells [9].

Low-level light therapy (LLLT) has long been used for improvement in local circulation. LLLT also enhances tissue healing by stimulating angiogenesis in various animal models of ischemia [10]. In addition, mesenchymal stromal cell proliferation, differentiation, and secretion of growth factors including VEGF and FGF are also enhanced by LLLT [11,12]. However, little is known about the therapeutic effect of LLLT on transplanted hASCs in animal models. This study was performed to determine the effect of LLLT on transplanted ASCs in a mouse model of ischemic skin flap. We compared the therapeutic angiogenesis effects between the ASC transplantation group and the ASC transplantation with the use of LLLT (ASC + LLLT) group.

Methods

ASC culture

hASCs were supplied from CEFO (Seoul, Korea) and were cultured in low-glucose Dulbecco's modified Eagle's medium F-12 (Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (Welgene), 100 units/mL penicillin and 100 μg/mL streptomycin at 37.0 °C in a 5% CO_2 incubator. hASCs between passages 5 and 8 were used for all experiments [13].

Fluorescence-activated cell sorting

Cells were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA). 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, USA), KDR (Beckman Coulter, Brea, CA), CD31 (Beckman Coulter), CD45 (Abcam, Cambridge, MA, USA), CD90 (BD Biosciences), CD105 (Caltac Laboratories, Burlingham, CA, USA) and CD29 (Millipore, Waltham, MA, USA). The cells were washed 3 times with PBS containing 0.5% BSA and resuspended in PBS for flow cytometry with the use of an Accuri device (BD Biosciences). Isotype control immunoglobulin (Ig)G was used as a negative control.

Histological staining

Samples were harvested 14 days after 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 to examine muscle degeneration and tissue inflammation. Masson's trichrome collagen staining was performed to assess tissue fibrosis in ischemic regions. The criteria used for the histological scores of skin flap were modified from previous reports [14] and are summarized in Supplementary Table I. The histological parameters were dermal regeneration and angiogenesis. The regeneration of skin appendages was assessed by counting the number of hair follicles or sebaceous glands in the skin flap.

Immunofluorescence staining

Indirect immunofluorescence staining was performed by use of 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 then were washed extensively in PBS. The sections were stained with specific primary antibodies and fluorescent-conjugated secondary antibodies (Supplementary Table I) with the use of a M.O.M. kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The cells were counterstained with DAPI (4,6-diamino-2-phenylindole dihydrochloride; Vector Laboratories). Negative control mouse IgG (Dako, Carpinteria, CA, USA) and rabbit IgG (Dako) antibodies were used as negative controls. To detect transplanted human cells, sections were stained with an immunofluorescent anti-human nuclear antigen (HNA, Millipore). Stained sections were viewed with the use of a model DXM1200F fluorescence microscope (Nikon, Tokyo, Japan). Processed images were analyzed for fluorescence intensity with the use of ImageJ software (NIH).

Western blot analysis

Samples were solubilized in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetra-acetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin and 2 μg/mL aprotinin) for 1 h at 4°C. Lysates then 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 means of SDS polyacrylamide gel electrophoresis with the use of 10% or 15% resolving gel and then were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody for 1 h at room temperature. For

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