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 angiogenesis 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...
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% CO₂ 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), CD105 (Caltac Laboratories, Burlingame, CA, USA), CD90 (BD Biosciences, 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-diamidino-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-acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1 mmol/L phe- nylmethylsulfonyl fluoride, 1 µg/mL leupeptin and 2 µg/mL aprotinin) for 1 h at 4 °C. Lysates then were clarified by centrifugation at 15,000g 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
Preparation of the experimental animal model

Experiments involving mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All aspects of the animal care and experimental protocols were approved by the Dankook University Committee on Animal Care. Seven-week-old male BALB/c mice (Narabio, Seoul, Korea) were anesthetized with Zoletil (30 mg/kg). A cranially based 4 × 2 cm flap with the flap base 1 cm caudal to the occipital neckline was elevated on the back. A 0.13-mm-thick silicone sheet was inserted to separate the flap and avoid neovascularization from the bed, and the flap was sutured in place.

Detection, peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were used as described by the manufacturer. Membranes were scanned to create chemiluminescent images and were quantified with the use of an image analyzer (Kodak, Rochester, NY, USA).

Enzyme-linked immunosorbent assay for angiogenic growth factor production

Angiogenic growth factor production in the L-spheroid was assayed with the use of a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Ltd, Abingdon, United Kingdom) according to the manufacturer’s protocols. Concentrations are expressed as the amount of angiogenic growth factor per 10⁴ cells at a given time.

Human angiogenic protein analysis

To analyze the expression profiles of angiogenesis-related proteins, we used the Human Angiogenesis Array Kit (R&D Systems, Ltd). Cell samples (5 × 10⁶ 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. After a wash step to remove unbound material, streptavidin-horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and enhanced chemiluminescence detection reagents were added sequentially. The signals on membrane films were detected by scanning on an image reader LAS-3000 (Kodak, Rochester, NY, USA) and quantified with the use of MultiGauge 4.0 software (Kodak). The positive signals seen on developed film were identified by placing a transparency overlay on the array image and aligning it with the two pairs of positive control spots in the corners of each array.

Low-level light therapy

The LED (WON Technology, Daejeon, Korea) was applied for 10 min daily from day 1 to day 20. The distance from the LED to the ischemic hind limb was 8 cm. This LED model had an irradiated wavelength of 660 nm and power density of 50 mW/cm². The fluence of each skin site was 30 J/cm² (1 mW × second = 0.001 J).

Statistical analyses

All the quantitative results were obtained from triplicate samples. Data are expressed as a mean ± standard deviation. Statistical analysis was carried out with the use of a two-sample t-test for comparing two groups of samples and one-way analysis of variance for three groups. A value of P < 0.05 was considered to be statistically significant.

Prevention of skin flap necrosis by use of ASCs with LED phototherapy

The animals were randomly distributed into 4 groups: the PBS group, the LLLT-treated group, the ASC-treated group and the combine treated group (ASC + LLLT). Two groups (ASC group and ASC + LLLT group) received hASCs (1.5 × 10⁶ cells; hASC group, n = 9 per group) cultured on TCP mixed with PBS (300 µL), which were then injected intramuscularly into 3 sites of the skin flap. The PBS group received an injection of PBS (PBS group, n = 9). An equivalent number of cells were injected in both conditions. Additionally, the LLLT group (n = 9) received light-emitting diode (LED) treatment. The physiological status of the ischemic skin flaps was followed for 2 weeks after treatment. Flap survival was estimated by measuring the sizes of the viable and ischemic areas with the use of digital image analysis. Images of the flaps at the same distance and on the same focus were taken by use of a digital camera. The results were expressed as percentages of survival area relative to the total flap surface area. The viable tissue was demarcated grossly in a blinded fashion on the basis of texture, color and appearance by two different observers. The survival area was calculated in square centimeters by use of Adobe Photoshop 7.0 (Adobe Systems, Inc, San Jose, CA, USA). Cutaneous blood flow was measured by use of a laser Doppler flow-meter (Laser Doppler Perfusion Imager System PeriScan PIM 3 System; Perimed AB, Stockholm, Sweden). Before scanning was initiated, mice were placed on a heating plate at 37°C. Euthanasia was conducted by intravenous injection of thiopental sodium (40 mg/kg).
Figure 1. Enhanced expression of hypoxia-induced survival factors and angiogenic growth factors in hASC + LLLT groups. hASC + LLLT and hASC were cultured for 3 days. (A) Western blot analysis and quantification of HIF-1α in hASCs. (B) Enzyme-linked immunosorbent assay measurement of hASCs cultured for 3 days. Concentrations of growth factors are presented as pg corrected for 10^4 cells (*P < 0.05, compared with 6 J/cm^2 group, t-test, n = 3 in each group). (C) Angiogenesis-related protein analysis of hASCs.
Results

Characterization and phenotype of hASCs

Adherent cells obtained from human adipose tissue were grown in vitro. The cells were positive for human MSC markers CD29 (β1 integrin; 96.9%), CD90 (Thy-1; 96.9%) and CD105 (endoglin; 70.9%). However, the cells were negative for human endothelial cell markers CD34, CD31 and KDR (VEGF receptor) and hematopoietic cell marker CD45 in immunofluorescence staining and flow cytometry analyses (Supplementary Figure 1A,B). These results indicate that the expanded cells included a large population of hASCs and that they were not contaminated with endothelial cells.

Production of angiogenic factors by hASCs

The ASC + LLLT group showed an increase in the expression of hypoxia-induced survival factors such as hypoxia-inducible factor (HIF)-1α, when compared with cells in not grown in LLLT-treated culture (Figure 1A). The hASC + LLLT group showed considerable expression of the angiogenic growth factors hepatocyte growth factor and VEGF and fibroblast growth factor 2 (FGF2) (Figure 1B). The expression of angiogenic growth factors (bFGF, VEGF and hepatocyte growth factor) in the ASC + LLLT group was much greater than in the ASC group (Figure 1C).

Survival of ASCs in the skin flap

At 14 days, apoptotic factor caspase 3–positive cells and HNA-positive cells were identified by means of fluorescence microscopy throughout the ischemic tissue to determine whether locally transplanted ASCs were incorporated into the ischemic skin flap. In the ASC and ASC + LLLT groups, ASCs were observed in the regenerated ischemic tissue (Figure 2A). The ASC + LLLT group exhibited significantly increased numbers of HNA-positive cells (ASC group: 12%; ASC + LLLT group: 33% per DAPI-positive cells) (Figure 2B) and decreased proportions of caspase 3–positive ASCs (ASC group: 47%; ASC + LLLT group: 11% per HNA-positive cells) (Figure 2C). The HNA+ cell–to–DAPI+ cell ratio of the ASC + LLLT group was 3 times higher than that of the hASC group.

Enhanced secretion of angiogenic growth factors from grafted hASCs

Transplantation of hASCs into ischemic tissue enhanced paracrine secretion of angiogenic growth

Figure 2. Survival of transplanted hASCs in skin flap. (A) In the hASC and hASC + LLLT groups, DAPI (blue) and caspase 3–positive cells (apoptotic marker; red) were detected after immunostaining at 21 days. hASCs were stained with HNA (green). Apoptosis of transplanted hASCs (arrows) was reduced in the hASC + LLLT group. (B) Ratio of HNA-positive cells (transplanted hASCs) to DAPI-positive cells (total cells) in the ischemic region (*P < 0.01, n = 4). (C) Ratio of caspase-3–positive cells plus HNA-positive cells (apoptotic transplanted hASCs) to HNA-positive cells (transplanted hASCs) in the ischemic region (*P < 0.01, n = 4).
factors. Double immunofluorescent staining of HNA and the angiogenic growth factors VEGF and FGF2 indicated secretion from transplanted hASCs in the ASC and ASC + LLLT groups (Figure 3A). Secreted human growth factors were mainly distributed in the vicinity of transplanted hASCs (HNA-positive cells). Compared with the ASC group, more growth factor-positive ASCs were observed in the ASC + LLLT group (Figure 3A). Western blot assay showed that significantly higher levels of VEGF and bFGF were secreted by the LLLT and ASC + LLLT groups than by the PBS group, and greater amounts of growth factor were observed in the ASC + LLLT group than in the ASC group or the LLLT group (Figure 3B). However, there was no significant difference between ASC-treated tissues and PBS-treated tissues to indicate that the ASC + LLLT group was more effective than the ASC group at increasing transplanted cell retention and VEGF expression.

Angiogenic efficacy

To verify the angiogenic effect of transplanted cells, the number of arterioles was analyzed (n = 10 in each group; Figure 4A,B). Many of the human CD31+ cells in the ASC + LLLT group were double-stained for smooth muscle actin. Endothelial cells and perivascular cells that differentiated from the injected human cells were detected by use of α-smooth muscle actin and hCD31 antibodies, respectively (Figure 4A,B). The number-per-unit area (1 mm²) of CD31+ vessel-like structures was compared among groups, as shown in Figure 4B. The area of the ASC + LLLT group increased to 5 times that of the ASCs group. Because we observed increased vessel formation in the ischemic tissue and, because it is well known that the angiogenic process is stimulated by hypoxia, we further tested the hypothesis that the beneficial effect of LLLT could be due to activation of the transcription factor HIF-1α). A Western blot assay demonstrated significantly higher levels of HIF-1α secreted by the LLLT and ASC + LLLT groups than by the PBS group (Figure 4C), and greater amount of growth factors in the ASC + LLLT group than in the ASC group. However, there was no significant difference between ASC-treated tissues and PBS-treated tissues. HIF-1α could be responsible, at least in part, for some of the beneficial effects of LLLT therapy on ischemic tissues. These
findings suggest the greater effectiveness of ASC + LLLT treatment in angiogenesis in ischemic limbs.

Improvement of skin flap survival by transplantation of ASCs

The therapeutic potential of ASCs was evaluated by physiological observation of the ischemic skin flap 14 days after treatment. Our data showed that the ASC + LLLT treatment group had an accelerated healing of the skin flap (Figure 5A,B). At 2 days and 7 days after surgery, the ASC + LLLT group had significantly smaller necrotic areas than did the other groups. No significant difference was observed between the ASC and LLLT groups at any time (Figure 5A,B). At 14 days, all of the skin flaps of the ASC + LLLT group achieved almost complete survival, whereas the skin flaps of the other groups did not reach the same level of survival.

The relative blood flow images of the quantitative analysis of laser Doppler blood perfusion image ratio are presented in Figure 5C,D. The increased blood flow index showed that mice receiving PBS (the PBS group) were considerably impaired in terms of the severe ischemic damage resulting in the skin flap compared with the other groups. In particular, mice receiving ASC + LLLT treatment displayed normal skin tissue appearance, no auto amputation and a high degree of blood perfusion compared with the PBS or ASC treatment groups (Figure 5D). We observed increased vessel formation in the ASC + LLLT group (Figure 6B; arrows). Histological observation showed that the skin regeneration was much greater in the ASC + LLLT groups compared with the PBS group or the ASC group (Figure 6C). Our data indicated that the ASC + LLLT treatments enhanced re-epithelialization and granulation at 14 days (Figure 6A,C). In contrast, the ASC + LLLT therapy protected the skin flap from necrotic damage induced by ischemia (Figure 6A,B) and significantly reduced the rate of skin necrosis compared with the no-treatment group and the dissociated cell treatment group (Figure 5A,B).

Discussion

Endothelial progenitor cells and stem cells were previously examined for therapeutic angiogenesis in the treatment of ischemia [15]. Some transplanted ASCs expressed endothelial or smooth muscle markers and were incorporated into vascular networks in ischemic sites, but the frequency of incorporation was low because most cells died within the hypoxic microenvironment of the ischemic region [16,17].
Although the exact mechanism is unknown, the final pathway of the photobiostimulation process appears to be the modification of the gene response. Measurement of this response can be determined through gene expression and production of proteins that mediate inflammatory and healing responses. Thus, we decided to analyze the LLLT effect on the main transcription factor activated during hypoxia, HIF-1α. Cellular adaptation to the lack of oxygen is mediated by HIF-1α, which regulates the cellular response to physiological and pathological hypoxia by activating genes that are important to cellular adaptation and survival pathways under hypoxic conditions. This protein is stabilized under low oxygen conditions, whereas at higher oxygen environments, it is rapidly degraded by oxygen-dependent prolyl hydroxylase enzymes [9]. HIF-1α regulates the cellular response to physiological and pathological genes that are important to cellular adaptation and survival pathways under hypoxic conditions [18]. In our experimental model, we observed that the non-irradiated group expressed HIF-1α in response to tissue hypoxia (Figure 4C).

In the current study, ASC transplantation with LLLT significantly improved angiogenesis as well as the subsequent survival of the skin flap compared with transplantation of hASCs (Figure 2). This was probably caused by the enhanced survival of the ASCs (Figure 2), along with increased paracrine secretion (Figure 3). Specifically, enhanced paracrine secretion of angiogenic factors in the ASC + LLLT group at day 14 (Figure 3) could have been due to the enhanced cell survival (Figure 2). These data suggest that LLLT enhances the survival of ASCs by the inhibition of apoptosis. Furthermore, in the ASC + LLLT group, more VEGF- or bFGF-positive ASCs were observed in the regenerated tissue and greater amounts of growth factors were found in the skin flaps than in those of the ASC group. In addition, VEGF is one of the most important proangiogenic factors involved in therapeutic angiogenesis during and after ischemia [19].

Figure 5. Improvement of ischemic limb salvage by use of ASC + LLLT treatment transplantation. (A) Representative images of PBS, hASC, LLLT and hASC + LLLT treatment of skin flap on days 2, 7 and 14 after treatment. (B) Physiological status of skin flap 14 days after transplantation, n = 9 for each group. (C) Skin flap blood flow monitored in vivo by means of laser Doppler blood perfusion image ratio. In color-coded images, normal perfusion is depicted in red; marked reduction in blood flow of ischemic tissue is depicted in blue. (D) Blood flow score (*P < 0.05 compared with the hASC + LLLT group, n = 5 for each group).
bFGF is an important growth factor in the repair and regeneration of tissues because of the effects on migration and proliferation of fibroblasts, angiogenesis and matrix deposition [20]. In the irradiated groups, 660-nm light can increase HIF-1α expression in this model regardless of the fluence used. Of interest, increased HIF-1α expression (Figure 4) and the consequent induction of protein for VEGF occurred at the fluence of 660 nm (Figure 3). These data suggest that LLLT enhances the survival and functionality of the transplanted ASCs in the skin flap. LLLT effectively enhances the viability and proliferation of stem cells with the appropriate energy density and wavelength [21–23]. It can be concluded that HIF-1α induction after LLLT does not occur due to a negative effect on the tissue, because LLLT enhances the survival and functionality of the transplanted ASCs in the ischemic tissue [21–23]. HIF-1α expression is primarily induced by hypoxia, but its induction can also be mediated by growth factors and cytokines. In this case, the induction does not depend on the oxygen level but involves the activation of a different regulatory mechanism, possibly mediated by mitogen-activated protein kinase and the phosphatidylinositol 3-kinase/Akt signaling pathway [22]. Oxidative stress can also increase the expression of this transcription factor in the tissues.

Our study suggests that ASC transplantation with LLLT accelerates angiogenesis through differentiation and growth factor secretion. Furthermore, our results demonstrate that LLLT enhances the angiogenic effect of the ASCs by enhancing survival of the ASCs and stimulating the secretion of growth factors in the skin flap. In particular, the ASC + LLLT treatment enhances the functional recovery of the skin flap area with respect to the regeneration of tissues. These results may provide therapeutic approaches for the treatment of delayed regeneration of tissues.

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References


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