ORIGINAL ARTICLE

Effect of low-level laser irradiation and epidermal growth factor on adult human adipose-derived stem cells

B. Mvula · T. J. Moore · H. Abrahamse

Abstract The study investigated the effects of low-level laser radiation and epidermal growth factor (EGF) on adult adipose-derived stem cells (ADSCs) isolated from human adipose tissue. Isolated cells were cultured to semi-confluence, and the monolayers of ADSCs were exposed to low-level laser at 5 J/cm^2 using 636 nm diode laser. Cell viability and proliferation were monitored using adenosine triphosphate (ATP) luminescence and optical density at 0 h, 24 h and 48 h after irradiation. Application of low-level laser irradiation at 5 J/cm^2 on human ADSCs cultured with EGF increased the viability and proliferation of these cells. The results indicate that low-level laser irradiation in combination with EGF enhances the proliferation and maintenance of ADSCs in vitro.

Keywords Stem cells · Low-level laser · Epidermal growth factor · Viability · Proliferation

Introduction

Stem cells are defined as undifferentiated cells that can proliferate indefinitely and have the capacity for both self-renewal and differentiation to one or more types of specialised cells. Stem cells play a large role in basic biological processes in vivo, including the development of an organism and tissue repair. To date, remarkable progress has been achieved in the field of stem cell research—from their isolation and culture, to being used in genomic studies, drug discovery and cell-based therapy [1].

Adipose tissue is derived from the mesenchyme and contains a supportive stroma that is easily isolated [2], and provides an abundant source of multipotent cells [3]. The stem cell population in adipose tissue is responsible for the replacement of mature adipocytes within the tissue through the life time of an individual [4]. Adult stem cells can be isolated from adipose tissue and lipo-aspirates in significant numbers and exhibit stable growth and proliferative kinetics in culture [5]. Human adipose-derived stem cells (ADSCs) have been shown to differentiate into bone, cartilage, fat or muscle, making them a potential source for future tissue engineering applications in clinical settings [5].

Stem cells can be identified by their expression of certain genes and proteins. One such protein expressed on surface membranes of stem cells is Thy-1. Thy-1 is used as a marker for a variety of stem cells, including mesenchymal stem cells [6].

Laser radiation at different intensities has been shown to inhibit as well as stimulate cellular processes [7]. Studies on low-level laser irradiation and stem cells have shown that low-level lasers can change the metabolism of stem cells, increase adenosine triphosphate (ATP) production, and so increase migration [8]. Furthermore, low-level laser irradiation has also been shown to promote the proliferation of rat mesenchymal bone marrow and cardiac stem cells in vitro [9]. Since low-level laser irradiation has been found to increase the ATP levels and proliferation of cells, it could be used to stimulate the in vitro production of higher stem cell numbers. Furthermore, the addition of specific growth factors could enhance the differentiation of the stem cells into different cell types that could, in turn, be used in tissue engineering applications and reconstructive surgery. However, in order for this approach to be effective for use in

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tissue engineering, certain criteria need to be met. These criteria include that the cells of interest should be found in abundance; should be harvested by a minimally invasive procedure; and should have the capacity to differentiate into multiple cell lineages in vitro and then be able to be transplanted safely and effectively back into a host [10].

The growth factor, epidermal growth factor (EGF), has been found to play important roles in the regulation of cell growth, proliferation and differentiation. It is also involved in tumour proliferation, metastasis, apoptosis, angiogenesis and wound healing [11].

EGF has been shown to participate in the development and differentiation of skin appendages, tissue repair and modelling, and it can activate ectodermal and mesodermal markers [12, 13]. Furthermore, EGF has been found to increase cellular proliferation and viability in precursor cells of the central nervous system [14], as well as being able to induce the phosphorylation of extracellular signal-regulated kinase pathways. Studies on the effects of EGF on stem cells have found that pre-treatment of human ADSCs with EGF increased the differentiation potential of the cells along a neuronal lineage [15, 16]. Using yet another stem cell model, researchers have shown that EGF stimulates the differentiation of human mesenchymal stem cells into bone-forming cells [17]. Although EGF has the ability to block completely the accumulation of lipids in developing adipocytes, this has been associated with the potent stimulatory effect of EGF on cell proliferation [18]. To our knowledge, there have been, as yet, no studies on the effects of low-level laser irradiation in combination with EGF on ADSCs.

The aim of this study was to investigate the effect of low-level laser irradiation and EGF on adult stem cells isolated from human adipose tissue.

Materials and methods

Culture of ADSCs

Adipose tissue from consenting donors undergoing abdominoplasty was used for isolation of the adipose stem cells. Ethical approval for this study was obtained from the Academic Ethics Committee of the Faculty of Health Sciences of the University of Johannesburg.

Adipose tissue was separated from the dermal layer with scalpel blades. ADSCs were isolated from the tissue through enzymatic digestion, as previously described [19]. Briefly, adipose tissue was minced and incubated in collagenase type-1 solution. The dissociated tissue was then centrifuged, and the infra-natant was filtered through a 40 μm filter. The resultant cell suspension was used for cell culture. Isolated cells were cultured to semi-confluence in

Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, 21331-020, UK), 10% foetal bovine serum (FBS) (Delta Bioproducts, 14-501BI, South Africa), 0.1% penicillin/ streptomycin (Pan Biotec GmBH, Germany, PO6-07100), 1 μg/ml Fungizone (GIBCO, 15290-026) and 20 ng/ml EGF (Pan Biotec, CB-1101003). The cultures were incubated at 37°C in an atmosphere of 5% carbon dioxide (CO_2).

Laser irradiation

The diode laser (LTIO00-PLT20) 636 nm (Oriel, USA) used in this study was supplied and set up by the National Laser Centre (NLC, South Africa). Semi-confluent monolayers of ADSCs in complete medium with EGF (20 ng/ml) and without EGF were irradiated with the lids off the 3.3 cm^2 plates in the dark, at room temperature, with 5 J/cm^2 at 636 nm. Laser irradiation was delivered to the culture plate from above via an optical fibre. The beam was clipped to cover the entire area of the plate (3.3 cm^2). On average, a power output of 110 mW was measured, and this was calculated to take 6 min and 53 s to deliver 5 J/cm^2. The power density was 12.1 mW/cm^2. Non-irradiated cells were used as controls and were kept under the same conditions. Both irradiated and non-irradiated samples were re-incubated at 37°C in a humidified atmosphere of 5% CO_2.

Protein expression

We performed Western blot analysis to determine the expression of the stem cell marker, Thy-1 at 24 h and 48 h after irradiation without the addition of EGF.

Briefly, cultured cells were lysed in lysis buffer (equal volumes of Hanks' balanced salt solution (HBSS) and sample buffer—2 M Tris (hydroxymethyl) aminomethane, pH 6.8, BioRad, South Africa, 161-0719; 2% sodium dodecyl sulphate (SDS), BioRad, 161-0302; 100%glycerol, Separation Scientific, South Africa, 56-40-6, and H_2O) on ice. Cell extracts were sonicated, and protein concentration was determined with a bicinchoninic acid protein kit assay (BCA, Pierce, USA, 23228) [20].

Ten micrograms of protein were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to polyvinylidene diflouride membranes, (Immunoblot PVDF membrane, BioRad, 162-0177). Membranes were blocked overnight in blocking buffer containing Tris-buffered saline [TTBS—50 mM Tris; 150 mM sodium chloride (NaCl), Separation Scientific, 7647-14-5] containing 0.1% Tween 20 and 5% non-fat milk. This reduced background interference and prevented binding of the primary antibody to the membrane [21]. The membranes were then incubated in primary antibody (2 μg/ml,

1:100, Thy-1, Whitehead Scientific Group, South Africa, Sc-9163) diluted in blocking buffer (as above) at room temperature for 1 h. The membranes were washed in TTBS and then incubated in secondary antibody (0.2 μg/ml, 1:1,000, goat anti-rabbit horseradish peroxidase, Whitehead Scientific, sc-2004) diluted in blocking buffer (as above) at room temperature for 2 h. The membranes were washed as before and incubated in chemiluminescent substrate (Super-Signal West Pico, Pierce, USA, 34080) for 10 min protected from light. The blots were then exposed to X-ray film (Kodak MXG, Rochester, USA, 326052) for 2 min and 4 min. The films were developed and then viewed.

Cellular responses

We used cell suspensions of the cultured cells to measure cell viability through adenosine triphosphate (ATP) luminescence and cell proliferation through optical density readings.

Cell viability

Adenosine triphosphate luminescence

The CellTiter-Glo luminescent cell viability assay (Promega, South Africa, G7571) provides a homogeneous method for determining the number of viable cells in culture, based on the quantitation of ATP, which indicates the presence of metabolically active cells [22].

In accordance with the manufacturer's protocol, a mixture of cell suspension (500 cells/μl) in complete medium (50 μl) was mixed with an equal volume of Glo reagent (1 ml buffer and 0.007 g substrate) and mixed on a vortex for 2 min to induce lysis. The mixture (100 μl) was incubated for 10 min at room temperature and read on a luminometer (Hygiena International, Pi-102, Germany).

Cell proliferation

Optical density

We used optical density to measure cell proliferation. A hundred microlitres of cell suspension in complete medium (DMEM) was read at $A_{540\ nm}$ [23] in a microplate reader (Benchmark Plus Microplate spectrophotometer, Bio-Rad, USA).

Statistical analysis

All laser irradiation experiments and biochemical assays associated with measuring changes as a result of laser irradiation were performed six times ($n=6$), and each assay was performed in duplicate. The data were analysed with Sigma plot 8.0 software. Differences between groups were determined with Student's t-test for each independent variable, and one-way analysis of variance (ANOVA) was done on all the samples.

Results

Cell morphology

The morphology of the cells did not appear to have altered after the addition of EGF. The cells had maintained a smooth, elongated appearance (Fig. 1).

Protein expression

Western blot analysis revealed the expression of a stem cell marker, Thy-1, both 24 h and 48 h after irradiation in cells that had not been cultured with EGF (Fig. 2). Thy-1 expression was also observed in cells cultured with EGF and irradiated, both 24 h and 48 h after irradiation (Fig. 3).

Cell viability

ATP luminescence showed a significant increase in cells that had been cultured in the presence of EGF and irradiated in comparison with their respective controls that had not been irradiated or cultured in the presence of EGF at 0 h, 24 h and 48 h ($P<0.001$, $P<0.05$ and $P<0.001$, respectively) (Fig. 4). The increase was also significant in cells cultured with EGF but not irradiated in comparison with their respective controls that had not been irradiated or cultured in the presence of EGF, at all time points ($P<0.001$, $P=0.05$ and $P<0.001$, respectively). The increase in ATP luminescence in cells that had been cultured in the presence of EGF and irradiated compared with that in cells cultured with EGF but not irradiated was significant at 24 h and 48 h ($P<0.05$ and $P<0.01$, respectively); however, at 0 h there was no difference in ATP luminescence ($P=0.626$). Cells cultured with EGF and irradiated showed a significant increase in ATP luminescence in comparison with cells that had been irradiated and not cultured with EGF at 0 h and 48 h ($P=0.002$ and $P=0.006$, respectively), but, at 24 h, the increase was not significant ($P=0.109$). There was an increase in ATP luminescence in cells cultured with EGF and not irradiated compared with cells that had been irradiated and not cultured with EGF at 0 h ($P=0.007$); however, the increase was insignificant at 24 h and 48 h ($P=0.963$ and $P=0.254$, respectively). Irradiated cells cultured without EGF had shown an increase in ATP luminescence compared with that in control cells at 24 h and 48 h, but the increase was not significant ($P=0.321$ and $P=0.610$, respectively). There was a decrease in the

Fig. 1 Morphology of the ADSCs at 0 h, 24 h and 48 h. *A,* normal cells, not irradiated and not cultured with EGF. *B,* cells irradiated in the absence of EGF. *C,* cells not irradiated but cultured with EGF. *D,* cells irradiated in the presence of EGF. ×400
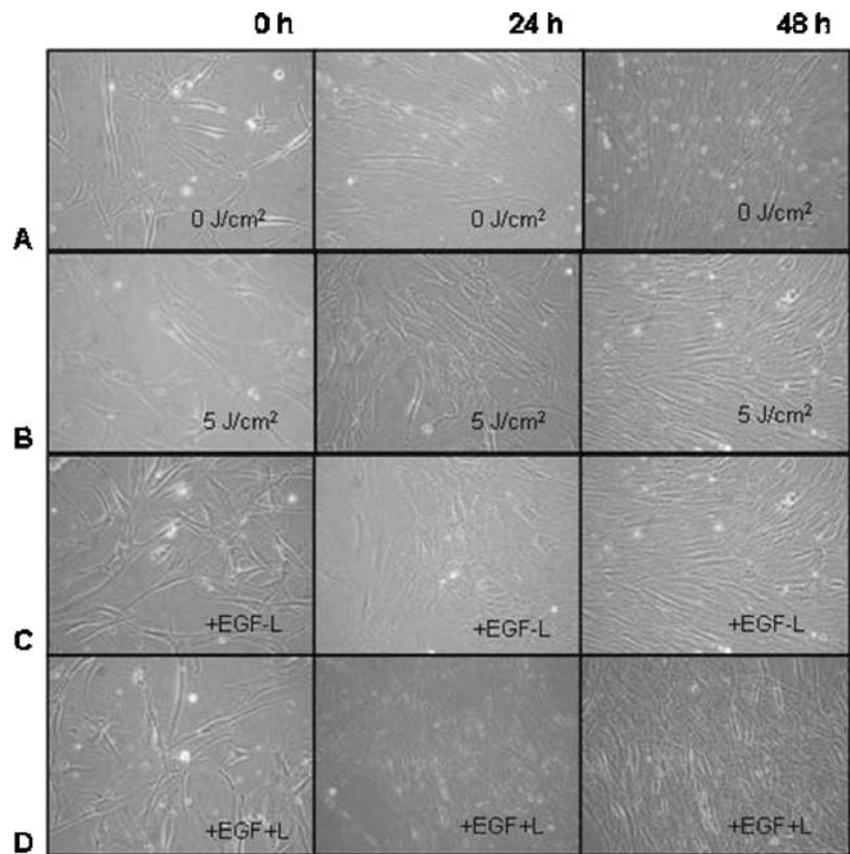
viability of cells that had been irradiated and not cultured with EGF compared with that in the control cells, although this decrease was not statistically significant. This might have been due to a technical error.

Cell proliferation

Optical density results showed an increase in cell proliferation in cells cultured with EGF and irradiated in comparison with their respective controls that had not been irradiated or cultured in the presence of EGF (Fig. 5). The increase was significant at 24 h and 48 h ($P<0.01$ and $P=0.001$, respectively); however, the increase was not significant at 0 h ($P=0.126$). Cells cultured with EGF and not irradiated also showed an increase in cell proliferation when compared with their respective controls. The increase was significant at 24 and 48 h ($P<0.05$) but not at 0 h ($P=0.161$). Cells cultured with EGF and irradiated showed a significant increase in cell proliferation in comparison with cells cultured with EGF and not irradiated at 48 h ($P=0.050$); however, at 0 h and 24 h the increase was insignificant ($P=0.792$ and $P=0.116$, respectively). The increase in cell proliferation in cells cultured with EGF and irradiated showed a significant increase at 48 h compared with cells that had been irradiated and not cultured with EGF ($P<0.005$), but, at 0 h and 24 h, the increase was insignificant ($P=0.95$ and $P=0.455$, respectively). Cells cultured with EGF and not irradiated showed an insignificant increase at all time points when compared with cells that had been irradiated and had not had EGF added ($P=0.121$, $P=0.668$ and $P=0.93$). The increase in cells that had
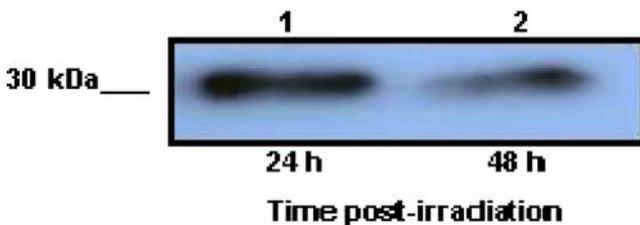

Fig. 2 Western blot analysis of the expression of Thy-1 in irradiated cells 24 h (*lane 1*) and 48 h (*lane 12*) after irradiation without the addition of EGF
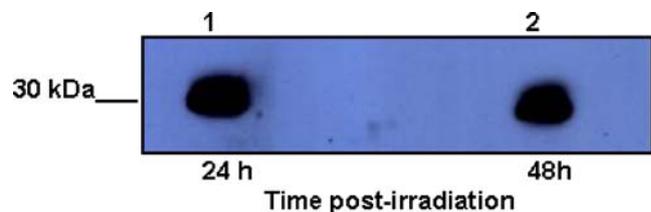

Fig. 3 Western blot analysis of the expression of Thy-1 in irradiated cells cultured with EGF at 24 h and 48 h after irradiation
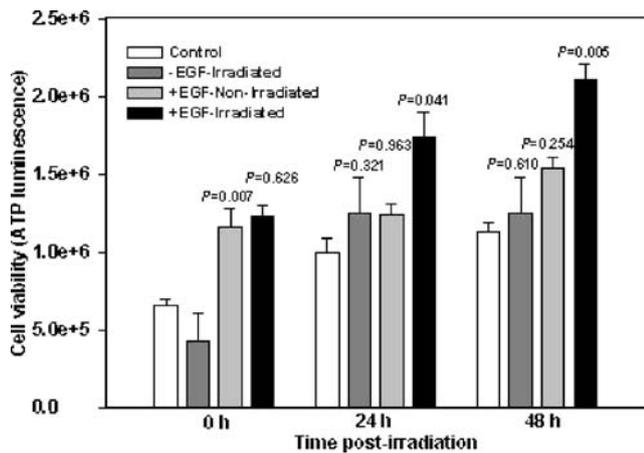
Fig. 4 We used ATP luminescence to determine cell viability of ADSCs at 0 h, 24 h and 48 h after irradiation. Cells not cultured with EGF were irradiated with 5 J/cm^2. Cells cultured with EGF were either irradiated with 5 J/cm^2 or not irradiated. Cells cultured without EGF and not irradiated were used as controls. P values are indicated for each time period after irradiation as compared with the individual controls, i.e. control compared with –EGF-Irradiated cells and +EGF-Non-irradiated compared with +EDF-Irradiated cells

been irradiated and not cultured with EGF in comparison with the control cells was also not significant at all time points ($P=0.938$, $P=0.101$ and $P=0.300$).

Discussion

In principle, adult stem cells are considered to be capable of self-renewal. It is thought that adult stem cells remain in an
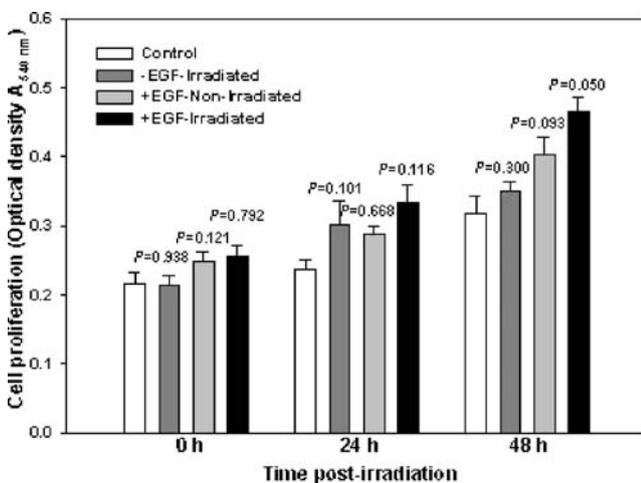

Fig. 5 We used optical density assay to determine cell proliferation in ADSCs at 0 h, 24 h and 48 h after irradiation. Cells not cultured with EGF were irradiated with 5 J/cm^2. Cells cultured with EGF were either irradiated with 5 J/cm^2 or not irradiated. Cells without EGF and not irradiated were used as controls. P values are indicated for each time period after irradiation compared with the individual controls, i.e. control compared with –EGF-Irradiated cells and +EGF-Non-irradiated compared with +EDF-Irradiated cells

undifferentiated state by suppression of intrinsic or extrinsic factors until stimulated to differentiate. Adult stem cells have been discovered and characterised in many tissues, and this suggests that these cells could be used for treatment of many diseases [24]. Diseases such as Parkinson's, stroke, multiple sclerosis, diabetes and traumatic injuries are caused by either a loss of cells or damaged cells. Adult stem cells could be used to treat these diseases and injuries. Adipose tissue contains an abundant, accessible, source of adult stem cells, and the stem cells provenient from this tissue are termed adult adipose-derived stem cells (ADSCs) [25, 26]. In our study ADSCs were isolated from human adipose tissue, and the effects of low-level laser irradiation alone, as well as in combination with EGF, were evaluated in vitro 0 h, 24 h and 48 h after irradiation. To date, no research conducted on the effect of low-level laser irradiation on ADSCs and in combination with EGF has been published.

In this study, Western blot analysis confirmed that the isolated and cultured cells were, indeed, stem cells by the expression of Thy-1, a stem cell marker known to be expressed by ADSCs. Thy-1 was expressed by the cells 24 h and 48 h after irradiation, whether cultured in the presence or absence of EGF.

Low-level laser irradiation has been found to promote proliferation and differentiation of human osteoblast cells in vitro at 632 nm with power output of 10 mW [27] as well as promote proliferation of mesenchymal and cardiac stem cells in culture at 1 J/cm^2 and 3 J/cm^2 [9]. Studies on low-level laser irradiation have shown that it could have a variety of biostimulatory effects, such as wound healing [28, 29], fibroblast proliferation [30–32], nerve regeneration [33], and collagen synthesis [34], and could increase migration of stem cells in vitro [8]

EGF as a growth factor plays a number of roles in organisms, including the regulation of proliferation and differentiation [35]. This growth factor is also involved in metastasis of cancer cells, programmed cell death, formation of blood vessels, wound healing and in tumour proliferation [11]. The addition of EGF to the cultures brought about an increase in cell viability and proliferation. This is similar to the results obtained in an experiment carried out by Svendsen et al. [14] in which EGF-supplemented cultures showed an increase in the numbers of precursor cells of the central nervous system that remained viable for longer periods than in cultures lacking EGF. Hauner et al. [18] also found that incubating stromal cells from human adipose tissue in EGF supplemented media had a potent stimulatory effect on cell proliferation; however, EGF also completely blocked the accumulation of lipids in these cells.

In our study the increase in cell proliferation was more pronounced in cells that had been cultured with 20 ng/ml of EGF and exposed to 5 J/cm^2 low-level laser irradiation than

in cells that had been cultured with the same concentration of EGF and not irradiated. The addition of EGF at different concentrations to other stem cell culture models has proven to be beneficial to the maintenance and proliferation of the cultured stem cells [36–38]. Indeed, it would be beneficial to the maintenance and expansion of ADSCs in culture if an optimal EGF concentration for this model could be determined; however, this requires further investigation.

Furthermore, this study showed that low-level laser therapy (LLLT), a treatment modality that involves the application of low-power monochromatic and coherent light in the treatment of numerous diseases ranging from chronic musculoskeletal aches and dermatitis to the treatment and of chronic cutaneous wounds in diabetic patients, is, indeed, a safe treatment and may actually stimulate adult stem cells in vivo to proliferate, which could aid in the healing process. However, the long-term effects of the exposure of stem cells to LLLT also requires further investigation.

ADSCs are considered to be a great source of stem cells for tissue engineering and regenerative medicine. The results presented here suggest that EGF in combination with low-level laser irradiation can increase the numbers and viability of cultured ADSCs. This is an important step in the expansion of stem cell numbers in vitro, especially in light of the potential role that ADSCs could play in regenerative medicine and tissue engineering, particularly in the use of autologous tissue transplants.

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