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Wound healing potential of adipose tissue stem cell extract

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ABSTRACT

Adipose tissue stem cells (ATSCs) are considered as a promising source in the field of cell therapy and regenerative medicine. In addition to direct cell replacement using stem cells, intercellular molecule exchange by stem cell secretory factors showed beneficial effects by reducing tissue damage and augmentation of endogenous repair. Delayed cutaneous wound healing is implicated in many conditions such as diabetes, aging, stress and alcohol consumption. However, the effects of cell-free extract of ATSCs (ATSC-Ex) containing secretome on wound healing process have not been investigated. In this study, ATSC-Ex was topically applied on the cutaneous wound and healing speed was examined. As a result, wound closure was much faster in the cell-free extract treated wound than control wound at 4, 6, 8 days after application of ATSC-Ex. Dermal fibroblast proliferation, migration and extracellular matrix (ECM) production are critical aspects of wound healing, and the effects of ATSC-Ex on human dermal fibroblast (HDF) was examined. ATSC-Ex augmented HDF proliferation in a dose-dependent manner and migration ability was enhanced by extract treatment. Representative ECM proteins, collagen type I and matrix metalloproteinase-1, are significantly up-regulated by treatment of ATSC-Ex. Our results suggest that the ATSC-Ex have improving effect of wound healing and can be the potential therapeutic candidate for cutaneous wound healing.

1. Introduction

Skin is the largest organ of the body, which is consists of the epidermis, dermis and subcutaneous layer, and protects us as a barrier from external environment [1–4]. The epidermis mostly consists of keratinocytes and major cell type of the dermis is fibroblast. The fibroblast is a cell that expresses and secretes the extra-cellular matrix (ECM), which is the major component of the dermis of the skin [5]. The ECM works to maintain homeostasis, to prevent skin aging, and to aid wound-healing [6]. Though the ECM is composed of various complex factors, most defining component of ECM is collagens. Most abundant types of the collagen is collagen type 1 (COL-1) [7], and its degrading enzyme is called the matrix metalloproteinases, also known as MMPs [8].

Since the skin is protective barrier against harmful environmental factors, its damage must be mended efficiently and properly. Repair efficiency is reduced by various factors such as aging, stress, diabetes and obesity, and impaired skin healing is a major area of unmet clinical need [9]. Wound healing process is consists of several different and overlapped stages including hemostasis, inflammation, tissue formation and tissue remodeling [9–12]. During inflammatory phase, neutrophils and macrophages infiltrate the wound area and produce cytokines to augment healing process. In the tissue formation and remodeling phase, the fibroblast migration, proliferation and ECM expression contribute to the process of skin repair. The expressions of ECM remodeling factors (COL-1 and MMPs) should be precisely controlled in these stages for perfect wound healing process [13,14].

Unlike other types of stem cells, adipose tissue stem cells (ATSCs) can easily be collected without ethical problems and can be differentiated into specialized cells [15–17]. Thus, they are studied as one of the leading sources for regenerative medicine research. Past research includes stem cell application for aiding tissue

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damage along with the most recent research done on paracrine function of stem cells, one of the many functions of stem cells. With the help of different growth factors and extra-cellular vesicles, ATSCs can play a key role in alleviating hostile environments. The secretion factors of stem cells have resulted in modulation of neurodegenerative diseases, ischemic damage, wrinkle-formation, wound-healing and hair growth [18–22]. In addition, a recent study suggests that the cell-free extract from stem cells can regulate various diseases.

Though the paracrine function is known to be one of the beneficial effects of fat stem cells, no research has yet been done on functions of fibroblasts for proper wound healing through ATSC extract (ATSC-Ex). In this research, we examined the effects of ATSC-Ex on wound healing *in vivo* and HDF functions *in vitro*.

2. Materials and methods

2.1. Ethics statement

This study using human samples was performed with approval from the Institutional Review Board (IRB) of the Seoul National University Hospital. All animal experiments were studied with the approval of the Institutional Animal Care and Use Committee (IACUC, Approval number: 13-0058-C2A1) of Seoul National University Hospital.

2.2. Culture of ATSC

Subcutaneous fat samples were obtained from normal humans who provided written informed consent to participate in the experiment. Adipose tissues isolated from the volunteers were kept in phosphate buffered saline (PBS) containing antibiotics (Invitrogen, CA, USA) and digested for cell isolation within a day. The adipose samples were digested in 0.075% collagenase type I solution (Invitrogen, CA, USA) with gentle shake for 1 h at 37 °C. Mature adipocyte fractions were removed from stromal fractions by centrifugation at 1200 × g for 10 min. The remaining stromal fractions were treated with red blood cell lysis buffer (Sigma), and centrifuged at 1200 × g for 10 min. The remaining stromal fractions of the samples were resuspended and seeded in endothelial growth medium−2 MV (EGM−2 MV; Clonetics, MD, USA), which contained vascular endothelial growth factor, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor−1, hydrocortisone, and ascorbic acid with 5% fetal bovine serum (FBS). The cells were used for the generation of ATSC-Ex after 3 or 4 passages.

2.3. Preparation of ATSC-Ex

For the preparation of extract of ATSC, the cultured ATSCs were harvested and centrifuged at 2000 × g for 8 min after washing twice with PBS. The ATSCs (approximately 4×10^7 cells in 175 T-flask) were suspended with 1 ml PBS and lysed by three cycles of rapid freeze/thawing. The lysate was centrifuged at 14,000 × g for 15 min, and the supernatant was passed through a syringe filter unit (0.45 μm) to remove cell debris. ATSC-Ex was freshly prepared just before use. All chemicals were purchased from Sigma. The total protein content of each ATSC-Ex was quantified using a bicinchoninic acid protein (BCA) assay kit (Pierce, IL, USA).

2.4. Protein extraction and western blot analysis

Cultured cells were washed then harvested in PBS using cell scraper. The preparation for protein extracts was done using RIPA buffer (Radio immunoprecipitation assay buffer, Thermo Scientific, Waltham, MA, USA) containing phosphatase and protease inhibitors (Roche, NJ, USA). Using BCA protein assay kit, the protein contents of cells were analyzed. Forty micrograms of protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 4–12% Novex NuPage Bis-Tris gel, Invitrogen, Mount Waverley, Australia) and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, MA, USA) in transfer buffer after blocking with 5% non-fat-dried milk which is dissolved in 1× TBST (Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature. The blots were incubated overnight at 4 °C with diluted primary antibodies. Blots were again incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:3000, GE Healthcare, NJ, USA), and developed using ECL solution (Enhanced chemiluminescence solution, Advansta, CA, USA). Band intensities were measured using IMAGEJ software from three independent results. Western blot figure show the representative one from three separate experiments.

2.5. Cell proliferation assay

Cell survival rates were measured by a colorimetric assay using the WST-1 (Roche, Mannheim, Germany) according to manufacturer's instruction. Briefly, cells were seeded in a 96-well plate and incubated with ATSC-Ex with varying concentrations, 0 μg/mL, 5 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL for 48 h. After the incubation period, WST-1 reagent was added to each well, and the cells were again incubated in 5% CO_2 at 37 °C for 2 h. Absorbance was measured using a plate reader at 450 nm (reference 650 nm) and the result shown represented the averages of three independent experiments.

2.6. Scratch assay

HDF was seeded using DMEM with 10% FBS in 24 well culture plates and maintained in 5% CO_2 at 37 °C for 24 h. A linear wound was generated using a sterile 100 μl pipette tip and cellular debris was washed with PBS. DMEM with ATSC-Ex or same volume of PBS was added and incubated for 12 h at 37 °C with 5% CO_2. Three representative images from each wells of the scratched area were taken using microscope to estimate migration ability of HDF. The experiments were repeated three times and migration distance from the wound edge was analyzed using ImageJ software.

2.7. In vivo wound healing assay

Circular cutaneous wounds were made using scissors on the back dermal skin of the mice: one on the left side of the spine, and the other on the right side of the spine. Amongst the two wounds, the right side wound was set as the experimental control group (topical application of ATSC-Ex 200 μg/200μl), whereas the left side wound was set as the control group (200μl of vehicle). After the wounds were made, each of the substances was applied to the corresponding wounds. Wound healing rates were recorded every two days over a period of 8 days. Every two days, the size was traced onto a transparent 3M paper. After the tracing, pictures of the wound size were taken to keep a visual record. After the 10 day experimental period, when the wounds were almost, if not all healed, the experiment ended.

2.8. Statistical analysis

All values indicated in the figures are represented as mean SE. Results of western blot were analyzed using Student's t-test. A two-tailed probability value below 0.05 was considered statistically