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Evaluation of a multi-layer adipose-derived stem cell sheet in a full-thickness wound healing model

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ABSTRACT

Cell sheet technology has been studied for applications such as bone, ligament and skin regeneration. There has been limited examination of adipose-derived stem cells (ASCs) for cell sheet applications. The specific aim of this study was to evaluate ASC sheet technology for wound healing. ASCs were isolated from discarded human abdominal subcutaneous adipose tissue, and ASC cell sheets were created on the surface of fibrin-grafted culture dishes. In vitro examination consisted of the histochemical characterization of the ASC sheets. In vivo experiments consisted of implanting single-layer cell sheets, triple-layer cell sheets or non-treated control onto a full-thickness wound defect (including epidermis, dermis, and subcutaneous fat) in nude mice for 3 weeks. Cell sheets were easily peeled off from the culture dishes using forceps. The single- and triple-layer ASC sheets showed complete extracellular structure via hematoxylin & eosin staining. In vivo, the injury area was measured 7, 10, 14 and 21 days post-treatment to assess wound recovery. The ASC sheet-treated groups' injury area was significantly smaller than that of the non-treated control group at all time points except day 21. The triple-layer ASC sheet treatment significantly enhanced wound healing compared to the single-layer ASC sheet at 7, 10 and 14 days. The density of blood vessels showed that ASC cell sheet treatment slightly enhanced total vessel proliferation compared to the empty wound injury treatment. Our studies indicate that ASC sheets present a potentially viable matrix for full-thickness defect wound healing in a mouse model. Consequently, our ASC sheet technology represents a substantial advance in developing various types of three-dimensional tissues.

1. Introduction

Wound healing that will enhance skin regeneration is clinically needed. Skin damage caused by burns, cuts, abrasions, and ulcers varies in the degree of severity [1]. Creating a viable skin substitute in vitro by assembling individual components is still challenging [2]. The limitation of current skin substitutes are poor vascularization, microbial contamination, lack of drainage, lack of mechanical strength, blistering, poor healing times and inadequate cosmetic effects [3,4]. Stem cells may create a complex structure that overcomes the limitations of individual skin substitutes due to their ability to differentiate into various tissue types by asymmetric replication [2]. Every stem cell division, self-renewing capacity, whereas the other enters a differentiation pathway and joins mature nondividing populations are described as asymmetric replaced special property of stem cells [5]. Among the main sources of cells that might be used for repair and regeneration of injured skin are embryonic stem cells, induced pluripotent stem cells and adult stem cells [6].

Adipose-derived stem cells (ASCs) and mesenchymal stem cells (MSCs) can self-renew and differentiate into various cell lineages [7]. ASCs derived from discarded human adipose tissue are immunocompatible and multi-potent, rendering them ideal for regenerative medicine applications, such as cartilage, bone, soft tissue and nerve repair [8]. Adipose tissue may be harvested from patients in a minimally invasive manner to provide a large quantity of autologous cells [9]. ASCs possess the highest proliferation and differentiation potential, followed by MSCs derived from bone marrow and cartilage [10]. Kim et al. [11] reported that ASCs promoted human dermal fibroblast proliferation and secretion by direct cell-to-cell contact. Furthermore, ASCs can be used to treat photoaging and wound healing. ASCs accelerated the wound healing rate and decreased the numerical density of fibroblasts in diabetic rats. The mechanism by which ASCs enhance the diabetic wound healing rate is unknown [12].

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Cell sheet technology has been used to enhance tissue-engineered organs in recent years [13]. The major advantage of using cell sheet technology in the tissue engineering field is customization and engineering of neo-tissue and an extracellular matrix (ECM) [13,14]. A temperature-responsive culture dish is used to make a viable cell sheet. The cell sheet can easily attach to other surfaces, such as culture dishes, other cell sheets and host tissues, due to an intrinsic ECM that is produced during in vitro culture [15]. Moreover, stem cell sheet technology has its own inherent potential for in vivo neovascularization [16]. Several studies have previously demonstrated the effects of cell sheets on wound healing [13,17,18]. In this study, we investigated the relationship of adipose stem cell sheets to wound regeneration. The use of a multi-layered cell sheet engineered in a critically sized athymic nude mice wound injury model was evaluated. Our results demonstrate that triple-layer ASC sheet treatment can significantly enhance wound healing.

2. Materials and methods

2.1. Cell isolation and cell sheet preparation

Adipose tissue (100–200 g) was harvested from the superficial abdominal depots of Caucasian females ($n = 3$) undergoing elective abdominal reduction surgery. The patient age range was 40–60 years old, and all were healthy according to clinical examination and laboratory tests. The University of Pittsburgh Institutional Review Board approved the adipose tissue sample collection procedure. Samples were not pooled, with each experiment using cells from three different patients. Abdominal adipose tissue was placed in a 50 ml centrifuge tube (10 g per tube) and immersed in a 1 mg ml^{-1} solution of collagenase. The isolated tissue was chopped with scissors and incubated at 37 °C for 35 min. The digested specimen was filtered, then centrifuged at 1000 rpm for 10 min. The pellet was suspended in erythrocyte lysis buffer. The solution was centrifuged again at 178 gravities for 10 min. The pellet, which contained mesenchymal stem cells, was cultured in Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were passaged at confluency and characterized as previously described by our laboratory [19].

We followed the modified commercial product protocol provided by Thermo Scientific Nunc Upcell Surface to fabricate the cell sheets. To create the cell sheet, 1×10^6 human ASCs were seeded onto a temperature-responsive cell surface plate (Thermo Scientific Nunc Upcell Surface) to a form a confluent layer. All medium was aspirated and PBS was added to prevent the cells from drying out. A fibrin-coated membrane was gently placed on top of the cell layer. The plate was incubated at 25 °C for 10 min. The cell layer was detached with sterile forceps and the membrane was transferred to another cell sheet layer with the attached cell layer facing downwards. The two attached cell sheet layers were cultured at 37 °C for at least 30 min, then 1 ml of the culture medium was added on top of the membrane and the membrane was gently withdrawn from the cell layer. The previous steps were repeated to create a three-layer ASC cell sheet.

2.2. Gene expression

RNA isolated during ASC cell sheet fabrication was used for quantitative polymerase chain reaction (qPCR). RNA was collected using a Qiagen RNeasy Mini Kit (Qiagen, USA). Approximately 200 ng of total RNA was reverse-transcribed into cDNA using a First Strand Transcription Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The PCR primers were designed using the Vector NTI (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. Efficiency was checked from tenfold serial dilutions of cDNA for each primer pair. A 2× SYBR® Green PCR Mastermix (Invitrogen, Carlsbad, CA, USA), 0.1 μM of each primer and the cDNA template were mixed in 25 μl volumes. qPCR was performed in triplicate in 96-well optical plates on Light Cycler 480 (Applied System, USA). β-Actin and mature adipocytes were used as inter-control and intra-control for qPCR analyses, respectively. The gene expression of mature adipocytes was normalized to 1.

2.3. In vivo studies

Athymic nude mice (6 weeks old, $n = 18$) were housed individually and received standard rat chow (Rodent laboratory chow 5001, Purina Co., USA) and water ad libitum in the Division of Laboratory Animal Resources at the University of Pittsburgh. All in vivo experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The mice were kept in a room with a constant temperature and a 12 h light–dark cycle. After a one-week acclimatization period, the animals were randomly divided into three groups: group 1: non-treated control (control) ($n = 6$); group 2: one-layer ASC cell sheet treatment ($n = 6$); and group 3: three-layer ASC cell sheet treatment ($n = 6$).

2.4. Surgical procedure

Each animal was weighed and anesthetized with ketamine (80 mg kg^{-1}) and xylazine (12 mg kg^{-1}) intraperitoneally. The surgical area cleaned with 70% ethanol. While the mice were anesthetized, a sterile technique was used to create two 12 mm diameter full-thickness surgical skin wounds to include epidermis, dermis and subcutaneous fat to the level of the panniculus carnosus on the back, which were standardized by using a 12 mm biopsy punch. Each mouse received the same treatment in both of his wounds. A donut-shaped silicone splint with a 12 mm diameter was centered on the wound injury and fixed to the skin using 5-0 nylon sutures (Ethicon Inc., Somerville, NJ) until day 21. The ASC cell sheet was applied to the wounds once immediately after wounding. Non-occlusive dressings were not used on the wound. The wounds were digitally photographed on days 0, 7, 14 and 18, and until the wound was completely healed (21 days). These time points were selected based on our preliminary results. The photographs were analyzed for healing progress, as assessed by changes in the wound surface area. At each time point, animals from each group were sacrificed and a 3 cm × 3 cm square was harvested from the center of each wound. The harvested wounds were fixed overnight in 4% paraformaldehyde at 4 °C for further analysis. An identical procedure was repeated for the triple-layer cell sheet wound treatment.

2.5. Histochemistry

Five micron sections were prepared from all paraffin skin blocks. The sections were deparaffinized with three immersions in xylene, then hydrated with descending concentrations of ethanol (100%, 90% and 70% to distilled water). Slides then were stained using different methods. A modified protocol was used to stain sections with hematoxylin & eosin (H&E) [20], Masson's trichrome [21] and Herovici's stain [22].

2.6. Human A/C protein

To identify human ASCs, sections of tissue were blocked with 5% horse serum for 1 h at room temperature. Human lamin A/C protein is a type V intermediate filament protein. Mouse anti-human lamin A/C (Vector Laboratories) in 2.5% horse serum stain reagent