Enhanced Epithelialization by Adipose-derived Stem Cells

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Abstract

Skin regeneration has improved significantly with the advent of bioengineered tissue. Previous studies showed that the use of dermal substitutes combined with autologous Adipose-derived Stem Cells (ASCs) improve angiogenesis and collagen synthesis; however, epithelialization is an outstanding issue to be resolved. In the present study, we evaluated the epithelial progression and differentiation in an extended period of time. We obtained ASCs from the inguinal fat pad of 4 Sprague Dawley rats. The non-expanded cells were cultivated in a commercially available dermal substitute for 48 hours and marked with a green fluorescent protein (GFP) lentiviral vector. The scaffold plus the stem cells and a cell-free scaffold (control) were implanted in the same rats. After 4 weeks, the epithelial surface was assessed by planimetry and histology. The wound closure by epithelialization was significantly higher in the ASC side (93.47% ± 5.98% vs. 79.88% ± 6.28%, P = 0.0028), and also linear epithelial advancement quantified by microscopy (6408 ± 275 μm vs. 5375 ± 250 μm, P <0.001). GFP positive cells were identified as part of the regenerated dermis but not the epidermis. The autologous ASCs seeded on a dermal substitute significantly increased epithelial formation, most likely by an induction mechanism rather than affecting differentiation.

Keywords: Epidermal epithelium; Stem cell; Adipose tissue; Artificial skin;

Abbreviations

AAPE: Advanced Adipose-Derived Stem Cell Protein Extract
ASC: Adipose-derived stem cells
bFGF: Basic fibroblast growth factor
BMSC: Bone marrow-derived stem cells
BSA: Bovine Serum Albumin
EGF: Epidermal growth factor

Introduction

Skin regeneration after full-thickness tissue injury is a challenge for the surgeon. The most commonly used option is a
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Material and Methods

Study Design

The study was carried out in four rats. In the first step, the ASCs were isolated from the inguinal fat pad and then seeded on scaffold of Integra® for 48 hrs, being marked with a lentiviral-GFP vector. In the second step, the scaffold with labeled ASCs was implanted in the same group of rats (autologous implantation) versus a control implant without cells. Four weeks later, the cicatrization behavior was evaluated by planimetry and histology to quantify the epithelialized surface and the destination of the labeled ASCs.

Animals and Surgery

Four male Sprague Dawley rats with an average weight of 280 g were used. All of the procedures were performed with the approval of the Bioethics Committee for animal research at the Faculty of Medicine, University of Chile.

Prior to surgical procedures, the animals were anesthetized using ketamine (80 mg/kg) and xylazine (8 mg/kg) intraperitoneally. The skin surface was washed with 2% chlorhexidine.

A 3 cm incision in the inguinal area was made to obtain the adipose tissue. Through subcutaneous dissection with Metzenbaum scissors, a sample of 1.4 g fat on average was taken. Hemostasis was performed, and the incision was sutured with nylon.

During the second surgical time, the resection of two opposed segments from the dorsal skin of 3.8 cm² was made, where the scaffold plus ASCs and the control were placed, suturing the implant with separate stitches (Figure 1).

Figure 1: Replacement of dorsum skin by Integra® scaffolds. The left corresponds to the ASCs side.

The animals were kept for 4 weeks in separate cages in a sterile work environment with conditioned air, and with ad libitum feeding. The silicone sheet that protects the implant was removed at day 7, to allow wound contraction and epithelialization.
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At the end of the study period, the rats were euthanized with an intraperitoneal overdose of sodium thiopental.

Cell Isolation

The adipose tissue sample obtained from the rat inguinal fat pad was fragmented and washed with phosphate-buffered saline (PBS). Then, enzymatic digestion with 0.01% collagenase type II was made (C6885; Sigma-Aldrich, St. Louis, Mo.) for 45 minutes at 37°C. After digestion, the suspension was neutralized with an equal volume of culture medium (Dulbecco’s Modified Eagle Medium and 10% fetal bovine serum and 1% antibiotics), passed through a 100 µm sterile filter; and then centrifuged at 1200 G for 10 minutes, resulting in a pellet with a high number of ASCs, referred to as Stromal Vascular Fraction (SVF) [18]. Generally, SVF contain 30-40% of ASCs along with endothelial, smooth muscle, and different blood cells [19,20].

Cell seeding on the dermal scaffold

3.8 cm² circular segments of Integra ® were placed in a 12 wells plate for culture with the silicone sheet side facing down. The wells were covered with 2 ml of culture medium with an average concentration of 1x10⁶ cells/ml.

In the second row of wells, the Integra scaffolds were similarly positioned to be used as controls, adding culture medium without ASCs.

The culture plate was left in an incubator at 37 °C and 5% CO2 for 48 hours; at the end of this period, only adherent cells remained in the scaffold, and the non-adherent cells were removed.

We previously determined the percentage of cell adhesion and viability of this technique by fluorescence of the CellTrace calcein on confocal microscopy and by MTT assay, viability of cells that adhered (Adherent, Adh) and did not adhere (Non-adherent, NA) to the implant, which was expressed as a percentage of the total number of cells in the culture. At 24 hrs, the viability of Adh cells was 70 ± 22% and after 48 hrs, the viability was 87% ± 21% [17].

Cellular infection with a lentivirus, for expression of a fluorescent protein and cellular tracking

After 24 hrs of culture, the obtained cells from a rat were transduced with a commercial lentiviral vector System (Biosciences, pGreenPure ShRNA scramble) using a multiplicity of infection of 1. The vector-containing medium was replaced prior to grafting.

Planimetric analysis to assess the percentage of cicatrization

At day 28 of the study, the rats were euthanized. The skin on the back was resected, extended over a flat surface and photographed with a ruler to quantify the surfaces using software Axiovision 4.8 (Carl Zeiss MicroImaging GmbH, Germany).

The wound closure percentage by contraction from the edges was quantified and the area inside the edges of the implant was measured, expressing the result as described below: [(grafted area on day 0 - day 28 area)/(grafted area day 0)] x 100.

Similarly, the epithelialization area was expressed as the difference between the end surface of the graft and the surface of the residual scab in the center of the implant (Figure 2A).

Histological study to evaluate epithelial advancement

After obtaining the tissue specimens, an incision was made just in the center of the lesion. Specimens were left for 48 hours in 10% formalin for tissue fixation and then embedded in paraffin. Then, serial sections of 5 microns thickness were made.

To quantify the migration of keratinocytes from the edges of the graft, the anti-cytokeratin antibody 34βE12 high molecular weight is used (M0630; 34βE12, 1:100, Dako Corporation, CA, USA). Epithelial advance quantification on the grafted area was made using Axio Vision software 4.8 (Figure 2B).

Histological immunofluorescence

Immunofluorescence analysis was performed to identify grafted ASCs with GFP signal and identify the phenotype of ASCs grafted cells by adding antibodies against additional antigens. The primary antibody was anti-cytokeratin 19 (ab15463, 1:100, Abcam, Cambridge, MA). Before adding the primary antibody, 5 μm frozen sections of fresh tissue samples were washed with PBS, then fixed with 3.7% paraformaldehyde for 10 minutes at room temperature, washed with PBS and 0.3% triton X-100 and submitted to blocking solution of PBS-BSA and 10% goat serum for 30 minutes at room temperature, washed with PBS, then fixed with 3.7% paraformaldehyde for 10 minutes at room temperature, washed with PBS and 0.3% triton X-100 and submitted to blocking solution of PBS-BSA and 10% goat serum for 30 minutes at room temperature. The primary antibody was incubated for 1 hour at 37 °C, then washed with PBS, along with the secondary antibody, and incubated for another hour (anti-rabbit Texas red IgG, 1:200, Vector Laboratories, Inc., CA, USA). Then, the slides were washed with PBS, aqueous mounting medium was added (S3025 Faramount, Aqueous mounting medium, Dako) and a cover glass was placed. The slides were analyzed in a fluorescence microscope.

Figure 2: (A) Planimetric analysis to quantify the healed wound epidermis. The darker central area represents the non-epithelialized area with scab and lighter peripheral the epithelialized area. (B) Anti-cytokeratin 34βE12 immunostaining and epithelial progress measuring. The upper lines represent the length of the quantified epithelium.

Statistical Analysis

Statistical analysis was performed using Stata software version 10.0 for Windows (StataCorp, Texas, USA). The results are expressed as the mean ± standard deviation. A t Student test was used for paired samples to determine the probability of statistical significance. A value of p ≤ 0.05 was considered statistically significant.

Results

Wound closure by planimetric analysis

The wound closure was quantified by planimetry of surface analysis. When analyzing the wound size by contraction from the edges, no significant differences were found between the ASCs and the control side (82.63% ± 3.4% vs. 80.66% ± 3.89%, p = 0.08); on the other hand, the epithelialized area was significantly greater in the ASCs than the control side (93.47% ± 5.98% vs. 79.88% ± 6.28%, p = 0.0028) (Figure 3).

Histology to quantify epithelial progression measured by immunohistochemistry anti-cytokeratin 34βE12

All histological samples were positive for cytokeratin antibody 34βE12. Linear microscopic quantization of the epithelium over the implant show that a significant difference exists in the ASCs side (6440 ± 485 μm vs. 5402 μm ± 467, P <0.001) (Figure 3).

Immunohistochemistry for tracking GFP labeled ASCs

At the end of the study period, the GFP positive ASCs were identified inside the regenerated dermis. However, there was no migration of these cells in the epidermal epithelium, based on detection of GFP activity concomitant with the surface marker anti-cytokeratin 19 (Figure 4).

Discussion

During skin wound re-epithelialization, a wedge-shaped mass of keratinocytes moves across the granulation tissue. The keratinocytes that participate in these processes of migration and proliferation are derived from at least two anatomic locations, including those cells in close proximity to the wound and epidermal cells of nearby hair bulges. Keratinocytes are stimulated to migrate and proliferate by a number of factors during re-epithelialization. Among growth factors and cytokines, Epidermal Growth Factor (EGF) and Transforming Growth Factor (TGF)-β play a role in keratinocyte migration [21,22].

EGF and others ligands bind the EGF Receptor (EGFR) a tyrosine kinase transmembrane protein. EGFR plays an important role in reepithelialization by increasing keratinocyte proliferation and cell migration in acute wounds. EGF is potent mitogen for keratinocytes and the transmembrane forms are able to stimulate growth of keratinocytes in a juxtacrine manner. In wound healing, TGF-β1 is important in inflammation, angiogenesis, reepithelialization, and connective tissue regeneration. During reepithelialization, TGF-β1 shifts keratinocyte integrin expression toward a more migratory phenotype [23].
The results of this study showed how epithelial proliferation successfully increased on the dermal scaffold plus ASCs in an animal experimental model over 4 weeks of regeneration. We used a brief technique for seeding ASCs without prior expansion (SVF), and in 48 hours, our previous results were supported in terms of adhesion and cell viability on the dermal scaffold [17]. The only technical modification in this time was the use of circular instead of square dermal substitute because the circular shape is geometrically adapted to the bottom of the culture dish, allowing adherent cells to be included in greater proportion in the scaffold

Regarding the quantification of wound healing, the measurement of the epidermal epithelium over the graft is very important, not only the wound size. Because the wound size mainly depends of the myofibroblasts activity around the edges, as reflected in the present study, no significant differences in wound size were found [21]. Other experimental studies that used ASCs carried on dermal scaffolds or on collagen gels found important differences for the closure of the wounds treated with ASCs but using the wound size as a measurement parameter [6,24,25].

In this study, the increased epithelialization cannot be explained by differentiation of ASCs in keratinocytes because at this time, no GFP activity was found in the epithelium. We are not in disagreement with the results presented by other studies that reflect, in a very illustrative way, how this differentiation exists at 4 weeks, but we must consider other alternatives to explain our results [6,7,24,26]. Rustad et al. recently postulated that MSCs (Mesenchymal Stem Cells) do not achieve transdifferentiation toward keratinocytes, but act by paracrine effects. The GFP marker was used in MSC, demonstrating the non-appearance of GFP+ MSCs in the healed wound epidermis [27]. They propose that structural microenvironments can significantly alter MSC behavior and can be used to manipulate their cytokine secretion profile. While the mechanism responsible for these upregulation cytokines and stemness factors is currently under investigation, a biostimulation between the delivery vehicle and ASCs that promotes epithelial formation is possible.

Yuan et al. exposed that the ASCs can promote the migration of Human Epidermal Keratinocytes (HEK) in vitro. The ASCs were directly co-cultured with HEK cells, and then confluent HEK cells were scraped to establish a wound model. After 72 hrs, the cell numbers that migrated across the edge of the wound was measured and the rate of wound healing was calculated. The number of cells were significantly higher and the wound healing was 61% significantly better than in a control group [28].

Moon et al. demonstrated the effects of Advanced Adipose-Derived Stem Cell Protein Extract (AAPE), obtained by the culture of ASCs in a hypoxic medium, on human keratinocytes in vitro, showing that AAPE in keratinocytes notably affected the expression of 290 identified transcripts, which were associated with cell proliferation, cycle and migration [29]. This treatment significantly stimulated stress fiber formation, which was linked to the RhoA-ROCK pathway, highlighting the importance of HGF, FGF-1, G-CSF, GM-CSF, IL-6, Vascular Endothelial Growth Factor (VEGF), and TGF-β3, among other relevant proteins.

Furthermore, hair growth was documented by paracrine biostimulation of ASCs [30,31]. Park et al identified the factors responsible for hypoxia-enhanced hair regeneration, finding increased proliferation of human follicle dermal papilla cells and epithelial keratinocytes, an increase in the insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, macrophage colony-stimulating factor (M-CSF), M-CSF receptor, platelet-derived growth factor-β, and VEGF, as reported with angiogenic and fibroblastic induction [25,30,32].

A last point to analyze is where to focus the benefits of faster healing. Logically, one goal is its use in situations, such as delayed healing, prolonged inflammation, low cellular infiltration, extracellular matrix reduction and reduction of growth factors, which occurs in diabetic foot [33]. However, in patients without healing problems, another goal should be to find a quicker method of skin regeneration, resulting in shorter hospital stays and fewer surgeries.

Conclusions

In this study, autologous Adipose-derived Stem Cells seeded on a commercially available dermal substitute significantly increased epithelial formation, most likely by a mechanism of induction rather than differentiation.

The simplicity of the performed method and the results provide support for clinical translation in the near future.

References

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