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Enhancing repair of full-thickness excisional wounds in a murine model: Impact of tissue-engineered biological dressings featuring human differentiated adipocytes

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ABSTRACT

Promotion of skin repair for acute or chronic wounds through the use of tissue-engineered products is an active field of research. This study evaluates the effects mediated by tissue-engineered biological dressings containing human in vitro-differentiated adipocytes and adipose-derived stromal cells (ASCs). Re-epithelialization, granulation tissue formation and neovascularization of full-thickness cutaneous wounds were specifically assessed using a murine model featuring a fluorescent epidermis. In comparison with wounds that did not receive an adipocyte-containing biological dressing, treated wounds displayed a slight but significantly faster wound closure based on macroscopic observations over 18 days. Non-invasive imaging of GFP-expressing keratinocytes determined that the kinetics of re-epithelialization were similar for both groups. Treated wounds featured thicker granulation tissues (1.7-fold, $P < 0.0001$) enriched in collagens (1.3-fold, $P < 0.0104$). In addition, wound cryosections labeled for detection of CD31-expressing cells indicated a 2.2-fold ($P < 0.0002$) increased neovascularization for the treated wounds at the time of terminal biopsy. This is in accordance with the secretion of pro-angiogenic factors detected in media conditioned by the dressings. Taken together, these results establish that a new type of engineered substitutes featuring a mixture of adipocytes and ASCs can promote cutaneous healing when applied as temporary dressings, suggesting their potential relevance for chronic wound management studies.

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1. Introduction

Chronic wounds and skin ulcers are major burdens for health care systems, in particular in the context of aging populations with an increasing proportion of diabetic patients [1]. During their lifetime, 15–25% of diabetic patients will develop at least one diabetic foot ulcer [2]. Among innovative methods developed to promote cutaneous healing, strategies relying on the therapeutic properties of mesenchymal stem/stromal cells (MSCs) are actively investigated (reviewed in [3]). Indeed, preclinical studies reported that MSCs derived from bone-marrow [4–6], cord blood and cord stroma [7,8], amniotic fluid [9,10] and adipose tissue (AT) [11,12] contribute to wound healing by acting upon the complex repair processes mediating wound closure, which comprise re-epithelialization, granulation tissue formation and angiogenesis. In particular, the paracrine effects of MSCs after their injection/implantation were reported to enhance repair in various contexts of tissue injury through the secretion of growth factors and angiogenic modulators [13,14].

Among MSCs, human adipose-derived stromal/stem cells (ASCs) are excellent candidates for cell-based therapies and tissue-engineering applications. Indeed, subcutaneous AT is abundant and easily harvested using a syringe or a minimally invasive lipoaspiration procedure.

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Regenerative strategies based on ASCs that have been described to improve skin wounds in preclinical studies include topical application of pre-conditioned medium [15], delivery of cell suspensions inside the wound beds [16] as well as intradermal injection of cells at the periphery of the wound margins [9]. Cellular approaches have investigated ASC's capacity to enhance repair of incisional [6], full-thickness [9,16] and ischemic [17] wounds. Tissue-engineered constructs comprising ASCs seeded into matrices such as silk fibroin–chitosan scaffolds [18], acellular dermal matrix grafts [19], and gellan gum–hyaluronic acid hydrogels [20] have also been evaluated.

AT itself has been associated with the improvement of skin conditions after fat grafting procedures to patients [21]. Medical case reports have revealed that subcutaneous lipoinjections could enhance the appearance of both mature and evolving scar tissues [22] and trigger healing of refractory chronic ulcers [23]. Although the precise mechanisms of action or the specific role that adipocytes may play are not fully understood, evidence from both preclinical [24–26] and clinical studies [27] points out that fat grafting could accelerate revascularization and reduce fibrosis surrounding the injection sites.

Using human ASCs, our team has previously demonstrated the production of manipulatable cell sheets and tissues using the self-assembly approach of tissue engineering [28,29]. Using this method, ascorbic acid and serum stimulate the cells to produce an endogenous collagen-rich extracellular matrix, leading to the formation of manipulatable cell sheets without exogenous or synthetic biomaterials. We also demonstrated that ASCs could be differentiated into adipocytes during cell sheet production, leading to reconstructed tissues featuring functional mature adipocytes embedded into human matrix containing a subset of ASCs that remained undifferentiated [28].

While implantation of ASC-containing cell sheets has been explored before [30–34], the use of adipocyte-containing biological dressings has not yet been investigated for wound healing applications. In this study, we postulate that such cellularized biological dressings produced using the self-assembly approach can be used in a temporary manner to promote endogenous skin repair mechanisms. Full-thickness splinted murine cutaneous wounds were treated with repeated applications of the biological dressings in comparison with untreated wounds of the control group. Interestingly, the specific investigation of re-epithelialization kinetics was achieved using a mouse strain featuring a fluorescent epidermis [35]. This K14–H2B–GFP mouse strain expresses Green Fluorescent Protein (GFP) conjugated to histone 2B (H2B) under the keratin 14 (K14) promoter: therefore their epidermal and follicular keratinocytes appear fluorescent. In addition to re-epithelialization, granulation tissue formation, neovascularization and the mechanical properties of the regenerated skin were also assessed. Our results indicate a beneficial impact of these novel tissue-engineered constructs featuring adipocytes and ASCs through enhanced granulation tissue formation and neovascularization.

2. Material and methods

2.1. Production of human tissue engineered dressings

All protocols were approved by the Institutional review board of the research center of CHU de Québec. Human adipose dressings were produced by tissue engineering using a cell sheet technology previously described [29], with minor modifications. Briefly, ASCs were extracted from lipoaspirated subcutaneous adipose tissue of a 38 year-old female donor of body mass index of 29.5. The adipose tissue was digested with 0.075% collagenase (type 1A, Sigma) in Krebs–Ringer Buffer for 60 min at 37 °C followed by 10 min with 0.25% trypsin. The stromal–vascular fraction was pelleted and a red blood cell lysis step in NH_4Cl was performed for 10 min at room temperature. The resulting cells were seeded at a density of 6.7–8.0 $\times$ 10^4 cells/cm^2 for in vitro expansion and batch cryopreservation after primary culture (passage 0). Fresh and thawed ASCs were cultured in Nunc culture flasks (Thermo scientific, Ottawa, ON, Canada) in expansion medium consisting of (1:1) Dulbecco's modified Eagle's medium (DMEM): Ham's F12 medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT, USA) and antibiotics (100 U/ml penicillin (Sigma) and 25 μg/ml gentamicin (Schering-Plough Canada Inc/Merck, Scarborough, ON, Canada)). For the production of the dressings, cells were seeded at passage 3 at a density of 2.34 $\times$ 10^4 cells/cm^2 in 12-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA), with media changes every two or three days. Each well contained a cell-sheet anchorage device allowing easy manipulation of the constructs (Whatman filter paper, GE Healthcare, Ottawa, ON, Canada) [29]. The expansion medium was supplemented with a 50 μg/ml freshly prepared ascorbic acid solution (AsA; Sigma–Aldrich, Oakville, ON, Canada) and was used for six days until adipogenic induction (Fig. 1A). In order to produce cellular sheets containing differentiated adipocytes from ASCs, cultures were exposed to a standard induction medium for 3 days, followed by the use of an adipocyte maintenance medium supplemented with AsA for the rest of the culture period [29]. The induction medium was composed of 100 nM insulin (Sigma), 0.2 nM T3 (Sigma), 1 μM dexamethasone (Sigma), 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) and 1 μM rosiglitazone (Cayman Chemical/Cedarlane, Burlington, ON, Canada) in 3% FCS-containing expansion medium. The adipocyte maintenance medium consisted of 10% FCS expansion medium supplemented with 100 nM insulin, 0.2 nM T3, and 1 μM dexamethasone (Sigma). Twenty-four days after seeding and 18 days after the induction of differentiation, the resulting adipose cell sheets were lifted and stacked in groups of three sheets to form a thicker adipose dressing. These constructs were maintained in culture until being used for animal experiments, as described in Fig. 1A.

2.2. Quantification of secreted molecules

Culture media conditioned by the adipose dressings for 24 h were assayed by enzyme-linked immunosorbent assay (ELISA) directed against human leptin, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and plasminogen activator inhibitor-1 (PAI-1) (DuoSets®, R&D systems, Minneapolis, MN, USA). Conditioned media (n = 4) were analyzed at two time points, namely after 31 and 52 days of adipogenic differentiation, respectively, representing the entire culture period during which the dressings were maintained in culture prior to their use in vivo (Fig. 1A).

2.3. Animal experiments

Homozygous transgenic K14–H2B–GFP founder mice were kindly provided by Dr Elaine Fuchs (Rockfeller University, New York City, NY, USA). Following a cesarean derivation, a colony was established in the animal facility of the research center of CHU de Québec (QC, Canada). All procedures involving animals were approved by the Institutional Animal Care and Use Committee. For wound healing studies, a silicone-splinted full-thickness wound model [36] was adapted in order to be compatible with the repeated applications of new dressings every three days. The use of silicone rings importantly reduces contraction upon wounding, allowing loose-skinned mammals to heal by secondary intention processes more reminiscent of human wound

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