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In vitro keratinocyte expansion for cell transplantation therapy is associated with differentiation and loss of basal layer derived progenitor population

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ABSTRACT

An alternative approach for traditional clinical mesh grafting in burn wound treatment is the use of expanded autologous keratinocytes in suspension or sheets that are cultured over 2–4 weeks in a remote service facility. While a wound reepithelialization has been described, the functional and aesthetic outcome is under debate. Cell isolation from split-skin donor tissue aims to preserve the valuable stem cell progenitors from the basal epidermal layer and to provide patients with a rapid wound re-epithelialization and a satisfying outcome.

While the presence of epidermal progenitors in the cell graft is thought to enable an improved epidermal surface post reepithelialization, we investigated a feasible clinical approach involving cultured versus noncultured epidermal cells comparing the $\alpha6int^{high}/K15^{high}/FSC^{low}/SSC^{low}$ and $\alpha6int^{high}/K5^{high}/FSC^{low}/SSC^{low}$ keratinocyte progenitor subpopulations before and after *in vitro* culture process. Our results show a significant increase of cell size during *in vitro* passaging and a decrease of progenitor markers linked to a gradual differentiation. A provision of the regenerative epidermal progenitors, isolated from the split-skin biopsy and applied directly onto the wound in an on-site setting of isolation and cell spray grafting in the operation room, could be of interest when choosing options for skin wound care with autologous cells.

1. Introduction

Traditionally, therapy for severe burns is based on surgical debridement followed by autologous split-skin mesh grafting (Struzyna and Krajewski, 2010). However, in extensive burns, the available donor area is limited and the treatments are associated with a high donor area burden. In larger burn-wound injuries, a lack of available skin for mesh grafting and rapid re-epithelialization can determine the chances of a patient's survival. Therefore, the development of new sources of autologous skin contributing to rapid reepithelialization and complementing mesh grafting is highly desirable.

Many technologies have been developed as alternatives to split-skin mesh grafting. These approaches include the use of dermal substitutes (such as, Integra, Life Sciences, Plainsboro, NJ) (Heimbach et al., 2003), decellularized cadaveric skin (Orgill, 2009), or more complex artificial skin that combines synthetic matrix with non-autologous cells (Supp and Boyce, 2005; Cooper et al., 1993; Naughton and Naughton, 1993) like Dermagraft (Dublin, Ireland) (Gentzkow et al., 1996) or Apligraft® (Organogenesis, Novartis, Canton, MA, USA) (Falanga and Sabolinski, 1999). A further approach is the use of *in vitro* expanded autologous keratinocytes cultured in remote service facilities over 2–4 weeks to cell suspensions for single cell spray-grafting or cell sheet grafting (Epicell, Genzyme, Cambridge, MA) (O'Connor et al., 1981; Compton et al., 1989; Gallico et al., 1984; Wright et al., 1998). Although a fast reepithelialisation was described when using cultured cells, the functional and aesthetic outcome, and the cost/benefit relationship of the *in vitro*

Abbreviations: ANOVA, Analysis of the variance; α6int, alpha 6 integrin; bkf, basal keratinocyte fraction; EPU, epidermal proliferative unit; FBS, fetal bovine serum; FDA, Food and Drug Administration; FSC, forward scatter; IFE, interfollicular epidermis; Kn, keratinocyte; Ker_f, whole keratinocyte fraction; P_n, Passage; PBS, Phosphate buffered saline; PGE2, prostaglandin E2; rhEGF, recombinant epidermal growth factor; rhIGF, recombinant insulin-like growth factor type-1; SC, stem cells; SSC, side scatter; TA, transit-amplifying cells

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culture procedure is under debate (Hata, 2007; Bello et al., 2001; Shakespeare, 2005).

Moreover, Rheinwald and Green (1975a, b) have reported in earlier papers that cultured keratinocytes could not completely generate a functional skin unless cultivated in combination with irradiated embryonic mouse derived fibroblasts. Because this product is a xenograft transplant, the Food and Drug Administration (FDA) impeded its commercialization limiting it to Humanitarian Use Device (HUD) (FDA, 2007). Therefore, an alternative cell expansion technology, without xenograft feeder layer, is the keratinocyte expansion in serum-free and low calcium medium combining growth factors and a collagen matrix to regenerate *in vitro* multilayer epidermis-like structures (Kim et al., 2004; Larderet et al., 2006; Izumi et al., 2007).

The main goal of our work is the establishment of keratinocyte procurement routines in combination with spray technologies suitable for clinical transition (Hartmann et al., 2007). An alternative to an on-site procurement routine in the operation room would involve epidermal skin cell isolation from a split-skin biopsy followed by *in vitro* cell expansion in low calcium and serum-free medium before cell spraying. In healthy skin, the epidermal basal progenitor cell populations enable a skin turnover every 39 days (Weinstein et al., 1984) by a succession of divisions and a programed differentiation process (Fig. 1). Therefore, the analysis of the basal cell subpopulations prevalence during the *in vitro* expansion process was of interest.

2. Material and methods

2.1. Skin tissue origin

The unidentified adult skin donations ($n=6$) were obtained under IRB exemption approval (0511186, University of Pittsburgh) from abdominal tissue reduction from patients at the Department for Plastic Surgery at the University of Pittsburgh Medical Centre (UPMC) in Pittsburgh, Pennsylvania. Prior to cell isolation, the specimens were exposed to a storage time of 2–3 h at 4 °C in phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA) solution containing 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 2.50µg/ml Amphotericin B (Invitrogen).

2.2. H and E histology on tissue samples

Pieces of the skin biopsies were embedded in polyvinyl-based medium Tissue-Tek® (Sakura Finetek, Torrance, CA, USA) and prepared for tissue sectioning by immersing them into nitrogen liquid and precooled in 2-methylbutane (Sigma-Aldrich, Saint Louis, MO, USA). After cutting and immersion into gradual series of ethanol and acetone, 3 µm sections were stained with hematoxylin and eosin (Bio-Optica, Milan, Italy). Light microscopy was performed using a Nikon Eclipse 50i microscope with a Nikon DS Fi1 camera and software for image acquisition (Nikon, Tokyo, Japan).

2.3. Keratinocyte isolation

The 0.2mm-thickness split-skin biopsies were obtained following transport into the laboratory by using a disposable dermatome (Teleflex, Limerick, PA, USA) under sterile conditions. The biopsy was then cut into small squares of approximately 6 mm x 6 mm using forceps and a scalpel placed into a tube of 37 °C prewarmed dispase-II-solution (Roche, Indianapolis, IN, USA). The tissue was then incubated for 40 min. at 37 °C to break down the connection between the dermis and epidermis. The epidermis was then placed into a 37 °C prewarmed Trypsin/EDTA-solution (0,05%/0,02%) (Gibco, Life Science Technologies, Grand Island, NY, USA) for 15 min. The reaction was stopped by adding 5% fetal bovine serum (FBS) (PAA Laboratories, Dartmouth, MA, USA) into the tube. Keratinocytes were sieved with a 40 mm strainer (CORNING, Corning, NY, USA) then washed with PBS and centrifuged at 200 g for 5 min.

2.4. Keratinocyte culture

After tissue processing, 3×10^5–10^6 keratinocytes were placed in 150 cm^2 collagen-coated flasks (Collagen I Cellware, *BD Biosciences, Bedford, MA, USA)* without feeder cells for expansion. Beyond passage 1, 3×10^5 keratinocyte were seeded in 150 cm^2 flasks. Standard cultures were maintained in a CO_2 incubator (Heraeus BB 6060, Kendro, CORNING, Corning, NY, USA) at 37 °C in a humidified atmosphere with 5% CO_2 air. After cell isolation and during passages, cells were cultured in EpiLife supplemented with 1% EDGS (rhEGF, rhIGF, PGE2, transferrin and hydrocortisone), 1% Penicillin/Streptomycin and 1% Amphotericin B (all Gibco products). The medium was changed every 2 days. As 70–80% confluence was reached, the cells were passaged with 0.05% trypsin and 0.2% EDTA (Gibco). Cell suspensions were counted using a Neubauer haemocytometer (Merck, Darmstadt, Germany). Culture quality control, microbiology testing, and morphology examination was done by phase-contrast microscopy with an Axiovert 25 microscope (Zeiss, Göttingen, Germany). For immunofluorescence staining on cultured cells, 4-well slides (Thermo Scientific, Rochester, NY, USA) were coated with 10 µg/cm^2 collagen I (Becton

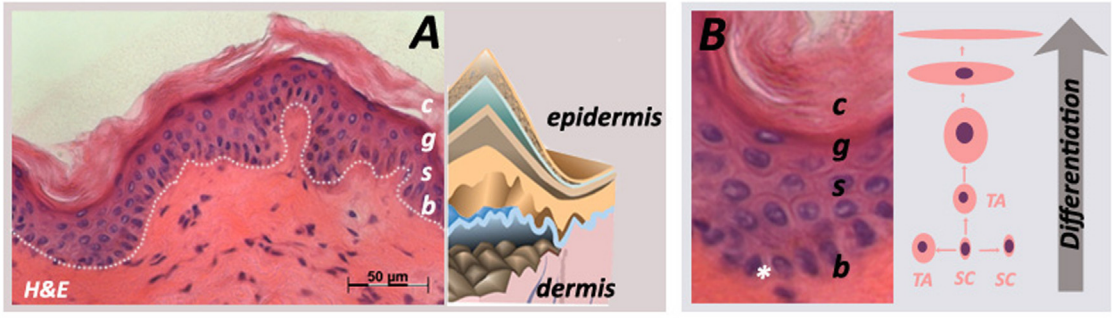

Fig. 1. Skin section with epidermal layers and the three-step-differentiation model. (A) H and E staining in light microscopy in a skin section showing the different epidermal layers. The dotted line shows the basal membrane that separates dermis to epidermis. Epidermis contains the layers, *stratum basale* (b), *stratum spinosum* (s), *stratum granulosum* (g), and *stratum corneum* (c). The contiguous Illustration (left) shows the skin tissue architecture in a drawing. (B) H and E staining section and contiguous drawing (left) shows the three-step model postulated by Fuchs (1995, 2008). with keratinocyte organization and differentiation process. Stem cells (*) generated from the *stratum basale* are self-renewed by symmetric divisions. Stem cells (SC) can also generate, by asymmetric divisions, transit amplifying (TA) cells with different commitment of differentiation that can replenish vacancies in the *stratum basale* or moving towards upper layers into the skin. The rest of the epidermis is constituted by the *stratum spinosum* (s) that contains active divisional TA cells, the *stratum granulosum* (g) with larger and differentiated keratinocytes, the *stratum lucidum* (not always present), and stratum corneum (c) with dead and packed keratinocytes forming the outer protective layer of the skin.