Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/burns

Study of proliferation and 3D epidermal reconstruction from foreskin, auricular and trunk keratinocytes in children

Jiad N. Mcheik [a,b,*], *Christine Barrault* [c], *Nathalie Pedretti* [c], *Julien Garnier* [c], *Franck Juchaux* [c], *Guillaume Levard* [a], *Frank. Morel* [b], *François-Xavier Bernard* [b,c], *Jean-Claude Lecron* [b,d]

[a] *Service de Chirurgie Pédiatrique, CHU de Poitiers, Poitiers, France*
[b] *Laboratoire Inflammation, Tissus Epithéliaux et Cytokines (LITEC), Université de Poitiers, Poitiers, France*
[c] *BIO**alternatives**, Gençay, France*
[d] *Laboratoire d'Immunologie et Inflammation, CHU de Poitiers, Poitiers, France*

ARTICLE INFO

Article history:
Accepted 6 July 2014

Keywords:
Burns
Children
Keratinocytes
Autografts

ABSTRACT

Objective: Severe burns in children are conventionally treated with split-thickness skin autografts or epidermal sheets. An alternative approach is to graft isolated keratinocytes. We evaluated foreskin and other anatomic sites as donor sources for autologous keratinocyte graft in children. We studied *in vitro* capacities of isolated keratinocytes to divide and reconstitute epidermal tissue.

Methods: Keratinocytes were isolated from foreskin, auricular skin, chest and abdominal skin by enzymatic digestion. Living cell recovery, *in vitro* proliferation, epidermal reconstruction capacities and differentiation status were analyzed.

Results: *In vitro* studies revealed the higher yield of living keratinocyte recovery from foreskin and higher potential in terms of proliferative capacity, regeneration and differentiation.

Cultured keratinocytes from foreskin express lower amounts of differentiation markers than those isolated from trunk and ear. Histological analysis of reconstituted human epidermis derived from foreskin and inguinal keratinocytes showed a structured multilayered epithelium, whereas those obtained from ear pinna-derived keratinocytes were unstructured.

Conclusion: Our studies highlight the potential of foreskin tissue for autograft applications in boys. A suitable alternative donor site for autologous cell transplantation in female paediatric burn patients remains an open question in our department. We tested the hypothesis that *in vitro* studies and RHE reconstructive capacities of cells from different body sites can be helpful to select an optimal site for keratinocyte isolation before considering graft protocols for girls.

* Corresponding author at: Service de Chirurgie Pédiatrique, Rue Jacques cœur, CHU de Poitiers, 86021 Poitiers, France. Tel.: +33 05 49 44 42 44; fax: +33 05 49 44 30 45.
E-mail address: j.mcheik@chu-poitiers.fr (J.N. Mcheik).

http://dx.doi.org/10.1016/j.burns.2014.07.003
0305-4179/

1. Introduction

Treatment of severe burns in children is conventionally achieved by the use of skin autografts for coverage. Disadvantages of this strategy include limited healthy donor sites in extensive burns and donor site morbidity. An alternative approach to address these drawbacks is to graft *in vitro*-expanded epithelial keratinocytes (CEA), using a reliable method of culturing human epidermal keratinocytes in stratified and coherent layers [1–5]. These autologous epidermal sheets have been successfully used, in addition to split-thickness skin grafts, in treating major burns [6]. Widespread use of these cultured epithelial autografts has been hampered by the long *in vitro* expansion times, sensitivity to infection, mechanical fragility of the sheets and labour-intensive process of preparing grafts, associated with the requirement of the relevant laboratory expertise [7–9]. In addition, clinical results have not been as satisfactory as expected, with reports of occurrences of hyperkeratosis and scar contracture [10–12]. Among the other alternatives to covering large areas of burns, the grafting of freshly dissociated keratinocytes on prepared burned skin presents the major advantages of simple handling of the keratinocyte suspension and a drastic reduction in the preparation time and cost [13–16]. This procedure has been developed by using an aerosol device to spray epithelial cells in combination with meshed split-thickness skin grafts, as previously reported in pigs [17,18]. In 2005, the standardized Recell device was introduced into clinical practice, thus allowing the immediate processing of small split-thickness biopsies to isolate keratinocytes. This technology has allowed grafting 80 cm^2 of burned skin from 1 cm^2 healthy tissue and has led to high quality scars, as compared to conventional skin grafting [19]. Today, the human scalp is proposed as the most commonly donor site for epidermal cells [20,21]. On another hand, we have shown that keratinocytes from foreskin have a high proliferation and full *in vitro* differentiation capacities [22]. Thus, in a previous pilot study, we successfully grafted male patients with autologous foreskin isolated keratinocytes that led to epithelialization and accelerated wound healing [23]. Recently, a multicentre trial has proposed a novel spray-applied cell therapy, containing growth-arrested allogeneic neonatal foreskin keratinocytes and fibroblasts, to heal adult chronic venous leg ulcers [24]. The purpose of the present study was to evaluate the regenerative capacities of keratinocytes isolated extemporaneously from several donor sites for autologous cell transplantation in burn children. We studied the *in vitro* capacities of isolated-keratinocytes to divide, differentiate and reconstruct epidermal tissue. Our studies could help in tissue selection with the prospect of grafting burns for girls.

2. Methods

2.1. Keratinocyte isolation and culture

Tissues were obtained from the Department of Pediatric Surgery (University Hospital of Poitiers), with the approval of the relevant ethical committee. Normal human epidermal keratinocytes were isolated from surgical samples of healthy foreskin from male children admitted for circumcision [$n = 40$, mean age = 4.2 (range 0.2–10) years] and 39 healthy other anatomic sites: posterior thin skin pinna admitted for otoplasty [$n = 19$, mean age = 7 (range 3–14) years] and trunk skin [$n = 20$, mean age 4 (range 0, 5–5 years)] [25]. Of the trunk skins, 10 biopsies were from inguinal skin. Briefly, the skin was rinsed in phosphate-buffered saline (PBS; Invitrogen, Cergy Pontoise, France) and subcutaneous fat was removed with scissors; the remaining tissue was cut into 2×0.5 cm skin pieces. Explants were treated overnight at 4 °C with dispase 2.5 U/ml (Invitrogen) in Keratinocyte-SFM medium (K-SFM; Invitrogen) supplemented with 25 μg/ml gentamycin sulphate (Sigma-Aldrich). After incubation, the epidermis was lifted off the dermis with forceps and transferred into 5 ml 0.05% trypsin (Invitrogen). The tissue was incubated at 37 °C/5% CO_2 for 15 min, with gentle trituration every 5 min. After centrifugation at $250 \times g$ for 10 min, the supernatant was removed and living cells were counted using a standard trypan blue (Sigma-Aldrich) exclusion assay in a Malassez chamber (R0). Cells were seeded in 175 cm^2 flasks in K-SFM/gentamycin supplemented with 25 μg/ml pituitary extract and 0.25 ng/ml epithelial growth factor (both from Invitrogen). The keratinocytes were then cultured for 7–10 days in order to reach the required 80% confluency, detached with trypsin as above, counted to estimate their proliferative capacity (R1) and used for *in vitro* experiments.

Reconstructed human epidermis (RHE, 3D) was prepared as previously described [26]. Suspensions of cultured keratinocytes (see above) were further cultured on polycarbonate culture inserts (Millipore) in EpilifeW medium supplemented with 1.5 mM calcium chloride and 50 μg/ml ascorbic acid, then transferred to the air–liquid interface for 5 or 12 days (D5, D12).

2.2. qRT–PCR

RNA extraction, quality control, quantification and reverse-transcription-real-time polymerase chain reaction (qRT–PCR) were performed as previously described [27]. For each marker, qPCR reactions were run on each of the samples. Primers (Sigma-Aldrich) were as follows: CASP14, (+) ATGCCTTGCACGTTTATTCC, (−) TTCAGGGTTCGTTTTCCTTG; FLG, (+) TGATGGTATTCAAGTTGGCTCA; (−) TGTTTCTCTTGGGCTCTTGG; CALML5, (+) AACTACGAGGAGTTCGCGAGGATG; (−) GCCCAAGGTCTGAAGGCAGAGAG; S100A7, (+) GCATGATCGACATGTTTCACAAATACAC; (−) TGGTAGTCTGTGGCTATGTCTCCC; KRT19, (+) AGCTCAATCTCAAGACCCTGAAGGG; (−) CAGGTCAGTGTGGAGGTGGATTCC.

2.3. In situ labelling

The primary antibodies used were anti-caspase 14 (NBP1-03128, Novus Biologicals; Littleton, USA), anti-filaggrin (SC-66192, Santa Cruz Biotechnology, Heidelberg, Germany), anti-S100A7 (IMG-409A, Imgenex, San Diego, USA) and anti-keratin 19 (MS-198-P, Thermo Scientific, Courtaboeuf, France). Species-specific secondary antibodies were conjugated to Alexa Fluor 488 (Life Technologies). Nuclei were counterstained with propidium iodide (Sigma-Aldrich). For immunofluorescence labelling of monolayer cultures, keratinocytes were fixed in