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Exploring the potentials of nurture: 2nd and 3rd generation explant human skin equivalents

Mogbekeloluwa O. Danso [a,b,1], Vincent van Drongelen [a,b,1], Aat Mulder [b], Gert Gooris [a], Jeroen van Smeden [a], Abdoelwaheb El Ghalbzouri [b], Joke A. Bouwstra [a,*]

[a] Department of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Leiden University, Leiden 2333 CC, The Netherlands
[b] Department of Dermatology, Leiden University Medical Centre, Leiden 2333 ZA, The Netherlands

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ABSTRACT

Background: Explant human skin equivalents (Ex-HSEs) can be generated by placing a 4 mm skin biopsy onto a dermal equivalent. The keratinocytes migrate from the biopsy onto the dermal equivalent, differentiate and form the epidermis of 1st generation Ex-HSEs. This is especially suitable for the expansion of skin material from which only small fragments of skin can be harvested *e.g.* diseased skin.
Objective: We evaluated whether 2nd and 3rd generation Ex-HSEs can also be generated from a single skin biopsy whilst maintaining the epidermal properties of 1st generation Ex-HSEs and native human skin.
Methods: 2nd generation Ex-HSEs were produced by placing a biopsy from the 1st generation Ex-HSE onto a new dermal equivalent. Likewise, the 3rd generation Ex-HSEs were generated from a 2nd generation Ex-HSE biopsy.
Results: We show for the first time that Ex-HSEs can be passaged to the 2nd and 3rd generation and display similar epidermal morphology and expression of differentiation markers as in native human skin and 1st generation Ex-HSEs except for involucrin. The 2nd and 3rd generation Ex-HSEs also show many similarities with 1st generation Ex-HSEs in lipid properties *e.g.* presence of all lipid classes, similar fatty acid chain length distribution and lamellar lipid organization. However, some differences arise in increased level of hexagonal lateral packing and a change in ceramide profiling. The changes in specific lipid classes were also accompanied by changes in the expression of the enzymes responsible for their synthesis.
Conclusion: The expansion of skin biopsies to the 2nd and 3rd generation Ex-HSEs could be a promising method to expand valuable epidermal tissue to analyze morphological and differentiation parameters in the native epidermis.

1. Introduction

Human skin equivalents (HSEs) are useful tools for studying the interplay between biological processes in the skin including modeling skin diseases [1–3], wound healing [4–6], cutaneous irritation and toxicity tests [7–9]. A novel method of generating HSEs involves placing full-thickness 4 mm skin punch biopsies onto a fibroblast populated collagen matrix *i.e.* dermal equivalent, forming the so-called 1st generation explant human skin equivalent (Ex-HSE) [3,10,11]. We previously demonstrated that the epidermis of 1st generation Ex-HSEs show comparable epidermal stratification and differentiation as the original *ex vivo* skin although the stratum corneum (SC) lipid properties could not be reproduced in Ex-HSEs [12]. Following this study, the next step

Abbreviations: Ex, explant; HSE, human skin equivalent; SC, stratum corneum; LPP, long periodicity phase; SPP, short periodicity phase; HPTLC, high performance thin layer chromatography; LC–MS, liquid chromatography–mass spectrometry; FTIR, Fourier transformed infrared spectroscopy; SAXD, small angle X-ray diffraction; FFAs, free fatty acids; SFAs, saturated free fatty acids; MUFAs, mono-unsaturated free fatty acids; CERs, ceramides; MTT, mid-point transition temperature; CerS, ceramide synthase.

* Corresponding author at: Department of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel.: +31 71 527 4208; fax: +31 71 527 4565.
E-mail address: bouwstra@lacdr.leidenuniv.nl (J.A. Bouwstra).
[1] These authors contributed equally to this work.

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was to investigate whether the amount of tissue generated from the 1st generation Ex-HSEs can be further expanded by growing a 2nd generation and even a 3rd generation Ex-HSE. In addition, since the free fatty acids play an important role in the changes in lipid organization in HSEs, we analyze for the first time in Ex-HSEs, the SC fatty acid chain length distribution and degree of saturation quantitatively. Furthermore, we examine the expression of some key enzymes involved in SC lipid synthesis because these factors contribute significantly to the SC skin barrier.

Expansion of epidermal tissue by producing up to three generations of Ex-HSEs can be crucial for analyses that require larger amounts of epidermal tissue, which cannot be harvested from patients with skin diseases. These analyses may include quantitative protein analysis or combining different techniques to study various aspects of diseased skin. By generating a 2nd generation Ex-HSE the total epidermal surface area can be expanded by 40 times compared to the original 4 mm skin biopsy (Table A1). The 1st generation Ex-HSE was generated from the original biopsy harvested from *ex vivo* skin. The 2nd generation was produced by harvesting a biopsy from the 1st generation Ex-HSE and implanting this onto a fresh dermal substrate. This approach can be repeated using a biopsy from the 2nd generation Ex-HSE to establish a 3rd generation Ex-HSE (Figure A1). During such organ culture, the keratinocytes migrate from the implanted biopsy onto the dermal equivalent, proliferate, differentiate and form an epidermis.

In native human skin, the SC, which is the outermost layer of the epidermis, consists of corneocytes embedded in a lipid matrix and this structure plays an essential role in the skin permeability barrier. The main lipid classes present in native human SC include free fatty acids (FFAs), ceramides (CERs) and cholesterol (CHOL). These lipids form two lamellar phases with repeat distances of approximately 6 nm and 13 nm referred to as short periodicity phase (SPP) and long periodicity phase (LPP) respectively [13]. Within the lipid lamellae, the lipids in native human SC are mainly organized in a dense orthorhombic packing although a fraction of lipids also adopt a hexagonal packing (Figure A2) [13–15].

The epidermal stratification, differentiation pattern and SC lipid properties from the 2nd and 3rd generation Ex-HSEs were analyzed in order to investigate whether these properties are similar to those in the 1st generation of Ex-HSE. In addition, the differentiation and barrier properties were also compared to the native human skin.

2. Materials and methods

2.1. Isolation of human dermal fibroblasts

Adult human breast or abdominal skin tissue was obtained from cosmetic surgery after written informed consent, according to the Declaration of Helsinki Principles. Dermal fibroblasts were isolated as described earlier [16] and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Netherlands) supplemented with 5% fetal bovine serum (FBS; Hyclone, UT, USA) and 1% penicillin/streptomycin (Sigma, Zwijndrecht, Netherlands) [17]. Passages 2–5 were used for the experiments.

2.2. Generation of human skin equivalents

Dermal equivalents were generated as described earlier [16,18]. Briefly, 1 mL of a 1 mg/mL collagen solution was pipetted into filter inserts (Corning Life sciences, Amsterdam, Netherlands). After polymerization, 3 mL of fibroblast populated (0.4×10^5 cells/mL) collagen (2 mg/mL) was pipetted on the polymerized collagen. After polymerization, the collagen was cultured submerged (1 week) in DMEM supplemented with 5% FBS, 25 mM ascorbic acid (Sigma, Zwijndrecht, Netherlands) and 1% penicillin/streptomycin.

The various generations of Ex-HSEs were generated as described in detail in Figure A1. These cultures consist of an explant and its outgrowing area. The explant is defined as the skin biopsy placed onto the dermal equivalent and the outgrowth is defined as the keratinocytes that after migration, proliferate and differentiate to form the main part of the Ex-HSE. After the generation of dermal equivalents, full thickness (FT) 4 mm fat free biopsies obtained from breast skin (referred to as the 1st generation explants, age 17–49), were gently pushed onto the dermal equivalents. Subsequently, the HSEs were cultured at the air-liquid interface and cultured for two days with DMEM and Ham's F12 (Invitrogen, The Netherlands) (3:1 v/v), 0.5 μg/mL insulin (Sigma, Zwijndrecht, Netherlands), 0.5 μM hydrocortisone (Sigma, Zwijndrecht, Netherlands), 1 μM isoproterenol (Sigma, Zwijndrecht, Netherlands), 1% penicillin/streptomycin, 25 mM ascorbic acid and 5% FBS. During the next two days, the HSEs were cultured in a similar medium as mentioned above with some changes. These included 1% FBS, 0.053 μM selenious acid (Johnson Matthey, Maastricht, The Netherlands), 10 mM L-serine (Sigma, Zwijndrecht, Netherlands), 10 μM L-carnitine (Sigma, Zwijndrecht, Netherlands), 1 μM α-tocopherol acetate (Sigma, Zwijndrecht, Netherlands), 25 mM ascorbic acid and a lipid mixture of 3.5 μM arachidonic acid (Sigma, Zwijndrecht, Netherlands), 30 μM linoleic acid (Sigma, Zwijndrecht, Netherlands) and 25 μM palmitic acid (Sigma, Zwijndrecht, Netherlands). For the remaining culture period, the HSEs were cultured with the same composition of the medium with two changes: (i) 7 μM arachidonic acid and (ii) no FBS. The culture medium was refreshed twice a week and the HSEs were cultured for 21 days at 37 °C, 90% relative humidity and 7.3% CO_2. At the end of the culture period, the dermal equivalent was covered with a keratinocyte outgrowth from the biopsy (1st generation Ex-HSE). During the harvest of the 1st generation Ex-HSE, a biopsy from the 1st generation outgrowth was placed onto a new dermal equivalent. This biopsy on the new dermal substrate is referred to as the 2nd generation explant and served as the start of the 2nd generation outgrowth. The Ex-HSE was cultured for another 21 days generating the 2nd generation outgrowth. During harvest, the 2nd generation outgrowth was further passaged to produce the 3rd generation Ex-HSE and cultured for 21 days. The fibroblast donor used to generate a series of 1st–3rd generations Ex-HSEs was kept the same.

2.3. Morphology and immunohistochemistry

Ex-HSEs were fixed in 4% (w/v) paraformaldehyde (Lommerse Pharma, The Netherlands), dehydrated and embedded in paraffin. For morphological analysis, 5 μm sections were stained with hematoxylin (2 mg/mL) and eosin (4 mg/mL). Immunohistochemical analysis for keratin 10 (K10), Ki67, filaggrin, loricrin, involucrin, steroyl-CoA desaturase (SCD) and ceramide synthase 3 (CerS3, analyzed by immunofluorescence) expression was performed as described in appendix materials and methods.

2.4. Lipid extraction/analysis

The SC was isolated from native human epidermis and HSEs as previously described [19]. The lipids were extracted from the SC using a modified Bligh and Dyer procedure [20]. SC extracts from the outgrowth of 2–3 Ex-HSEs of the same skin donor were pooled. The lipid composition of the explants could not be determined due to insufficient amount of material. Extracts were dried under a stream of nitrogen, reconstituted in chloroform: methanol (2:1) and stored at −20 °C. Lipid analysis by high performance thin layer chromatography (HPTLC) and liquid chromatography coupled to mass spectrometry (LC–MS) is described in appendix materials and methods.