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**Author:** Thakoersing, Varsha Sakina  
**Title:** Barrier properties of human skin equivalents : rising to the surface  
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MODULATION OF BARRIER PROPERTIES OF HUMAN SKIN EQUIVALENTS BY SPECIFIC MEDIUM SUPPLEMENTS

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ABSTRACT

Previous studies demonstrated that our in-house human skin equivalents show the presence of all stratum corneum (SC) barrier lipid classes, but show a reduced level of free fatty acids, of which a part is mono-unsaturated. In this study we aimed to improve the SC lipid properties of the Leiden Epidermal Model (LEM) by specific media supplements. For this purpose, the level of the fatty acid mixture (consisting of palmitic, linoleic and arachidonic acid) supplemented to the medium was increased four times or was modified by 1) replacing protonated palmitic acid with deuterated palmitic acid, 2) a fourfold increase in palmitic acid concentration or 3) addition of deuterated arachidic acid. Furthermore, the effect of insulin on the SC mono-unsaturated fatty acid content was studied. The results demonstrate that deuterated palmitic and arachidic acid are taken up into the LEM and are subsequently elongated and incorporated in the SC. Increasing the concentration of arachidonic and linoleic acid resulted in a decreased level of very long chain fatty acids and an increased level of mono-unsaturated fatty acids, while insulin did not affect the fatty acid profile. These results indicate that SC lipid properties can be modulated by specific supplements in the culture medium. This study therefore signifies the importance of medium composition on the SC lipid properties during culture of human skin equivalents.
INTRODUCTION

Human skin has a dual function: it prevents the entry of pathogens and harmful substances into the body and at the same time prevents excess water and electrolyte loss from the body. The permeability barrier of the skin is located in the extracellular lipid domains that surround the corneocytes in the stratum corneum (SC). The predominating lipid classes in human SC are cholesterol, free fatty acids and ceramides. The composition of these lipids in the SC plays an essential role in maintaining the permeability barrier.1-4.

In our previous studies we have demonstrated that our in-house human skin equivalents (HSEs) mimic many aspects of human skin, such as its morphology and the expression of early and late differentiation markers.5 Nevertheless, these HSEs showed a decreased permeability barrier compared to human skin when the diffusion of bezocaine was analyzed.5 Detailed examination of the SC lipids of the HSEs revealed that the total SC fatty acid content was reduced, while an increase in mono-unsaturated fatty acid (MUFA) content was observed (Thakoersing et al., submitted). Additionally, the SC of the HSEs contains ceramide species with a mono-unsaturated acyl chain. The observation of MUFAs and ceramides with a mono-unsaturated acyl chain indicates that both lipid classes have a common synthetic pathway.

In the previous studies the MUFA level in the SC of the HSEs was not quantified. It is therefore not known to which extent the MUFA level in the HSEs differs from native human SC and to which extent it affects the lipid properties of the SC. The altered fatty acid level and composition are expected to be the key factors for the presence of the mainly hexagonal packing in the HSEs compared to the dense orthorhombic packing observed in human SC.6-8 Optimization of the SC fatty acid level and composition of HSEs may therefore improve the SC barrier properties of HSEs to allow a better prediction of compound penetration through the skin.

Stearoyl-CoA desaturase (SCD) is an enzyme that catalyzes the biosynthesis of MUFAs from saturated fatty acids.9,10 The HSEs show the expression of SCD1 in
the basal and differentiated layers of the epidermis, while this protein is strictly localized in the basal layer of human skin (Thakoersing et al., submitted). The increase in MUFA content in the HSEs may therefore be attributed to the increased presence of SCD1 in the differentiated layers of the epidermis, which may result in an increased activity of SCD1. The culture medium used to generate HSEs is supplemented with a fatty acid mixture consisting of palmitic acid, arachidonic acid and linoleic acid. The supplementation of this mixture may influence the SC fatty acid level and composition in the HSEs. Furthermore, the activity of SCD is affected by a variety of factors, including insulin\(^9\)\(^{-12}\). Since the culture medium is also supplemented with insulin, this may influence the activity of SCD1 and therefore affect the SC fatty acid composition of HSEs. In this study we aimed to improve the fatty acid composition and organization in the SC of the Leiden Epidermal Model (LEM) by supplementing and altering the fatty acid and insulin levels in the culture medium. To determine whether the supplemented fatty acids are incorporated in the SC of LEM, protonated palmitic acid was replaced by deuterated palmitic acid. In some experiments the medium of LEM was additionally supplemented with deuterated arachidic acid. SC fatty acid profiling of LEM was performed by a novel LC/MS method. To our knowledge we report for the first time the effect of fatty acid supplementation on the SC lipid organization and composition in epidermal skin models.

**MATERIALS AND METHODS**

**Cell culture**

Normal human keratinocytes (NHKs) were established from mammary or abdomen skin, obtained from adult donors undergoing surgery, as described previously\(^{13}\). The Declaration of Helsinki principles were followed when working with human tissue. The NHKs were cultured with the Dermalife K medium complete kit (Lifeline Cell Technology, Walkersville, MD) and grown to 80%
confluency before trypsin digestion. First and second passage NHKs were used to generate LEMs.

**Generation of the Leiden Epidermal Model (LEM)**

LEMs were generated as described previously \(^5\) with minor changes. NHKs \((0.2 \times 10^6 \text{ cells/filter})\) were seeded onto cell culture inserts \((\text{Corning Transwell cell culture inserts, membrane diameter 12 mm, pore size 0.4 μm; Corning Life Sciences, Amsterdam, The Netherlands})\) and were kept submerged in Dermalife medium until confluency. Hereafter the HSEs were kept submerged in CnT medium \((\text{CellnTec, Bern, Switzerland})\), which was supplemented according to the manufacturer’s protocol, and 1% penicillin/streptomycin solution \((\text{Sigma, Zwijndrecht, The Netherlands})\), 0.25 μg/mL insulin \((\text{Sigma})\) and a lipid mixture consisting of 7 μM arachidonic acid \((\text{Sigma})\), 30 μM linoleic acid \((\text{Sigma})\) and 25 μM palmitic acid \((\text{Sigma})\). The LEMs were lifted to the air-liquid interface after two days and were cultured for an additional 12 days before harvesting. In order to improve the SC lipid properties of LEMs, modifications were made to the culture medium that was used during the time the LEMs were air-exposed. The specific modifications are provided in table 1.

**Immunohistochemistry**

Harvested HSEs were fixed in 4% \((\text{w/v})\) paraformaldehyde \((\text{Lommerse Pharma, Oss, The Netherlands})\), dehydrated and embedded in paraffin. 5 μm sections were cut and used for immunohistochemical staining of stearoyl-CoA desaturase 1 \((\text{SCD1; 100x dilution; Sigma})\), peroxisome proliferator-activated receptor α and β/δ \((\text{PPARα; 200x dilution and PPARβ/δ; 400x dilution; Acris Antibodies, San Diego, CA})\). The sections were deparaffinized and rehydrated with xylene and graded ethanol series and finally washed with PBS. The sections were immersed in sodium citrate buffer \((\text{pH 6})\) for 30 minutes close to the boiling point for antigen retrieval. Hereafter the sections were blocked with normal horse serum for 20 min
and incubated overnight at 4°C with the primary antibody. Next, the sections were incubated with the secondary antibody for 30 minutes, washed with PBS and incubated with ABC reagent for 30 minutes. The sections were consecutively washed with PBS, 0.1 M sodium acetate buffer and incubated for 30 minutes in amino-ethylcarbazole (Sigma) dissolved in N,N-dimethylformamide (1g/250 mL) (Sigma) supplemented with 0.1% hydrogen peroxide and finally washed with water. The sections were counterstained with haematoxylin. R.T.U. Vectastain Elite ABC Reagent Kit (Vector Laboratories, Burlingame, CA) was used for incubations with normal horse serum, secondary antibody and ABC reagent.

Table 1  Adjustment of the culture media

<table>
<thead>
<tr>
<th>Modification</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEM Control</td>
<td>FFA mix: 25 μM palmitic acid, 30 μM linoleic acid, 7 μM arachidonic acid</td>
</tr>
<tr>
<td>LEM C16:0D31</td>
<td>Replacement of protonated palmitic acid with deuterated palmitic acid (C16:0D31) (Isotec, Sigma)</td>
</tr>
<tr>
<td>LEM 4x FFA mix</td>
<td>Supplementation of FFA mix increased 4x</td>
</tr>
<tr>
<td>LEM 4x C16:0</td>
<td>Supplementation of palmitic acid increased to 100 μM (4x increase)</td>
</tr>
<tr>
<td>LEM + C20:0D39</td>
<td>Addition of 47μM deuterated arachidic acid (C20:0D39) (Cambridge Isotope Laboratories, Andover, MA) to FFA mix</td>
</tr>
<tr>
<td>LEM No insulin</td>
<td>No insulin added to culture medium</td>
</tr>
<tr>
<td>LEM 0.5x insulin</td>
<td>Supplementation of insulin (Sigma) lowered to 0.125 μg/mL medium (2x reduction)</td>
</tr>
</tbody>
</table>

Modifications made to the culture medium during generation of LEMs under air-exposed conditions. C16:0D31= deuterated palmitic acid with 31 deuterium atoms. C20:0D39= deuterated arachidic acid with 39 deuterium atoms.
Stratum corneum isolation

SC of LEM was isolated by overnight incubation on a 0.1% trypsin (Sigma) soaked filter followed by incubation at 37°C for 1 hour. The SC was separated from the viable epidermis and washed with a 0.1% trypsin inhibitor (Sigma) solution and water. The obtained SC sheets were dried and stored under argon. When using fresh human abdomen or mammary skin, fat tissue was removed and the skin was dermatomed to a thickness of approximately 300 μm. The SC was then isolated as described above.

Lipid extraction and HPTLC analysis

SC samples were extracted according to the Bligh and Dyer procedure with the addition of 0.25M KCl to extract polar lipids as described before 14, 15. The extracted lipids were analyzed by means of one-dimensional high performance thin layer chromatography (HPTLC) as described before 15. A standard lipid solution consisting of cholesterol, palmitic acid, stearic acid, arachidic acid, tricosanoic acid, behenic acid, lignoceric acid, cerotic acid and ceramides EOS, NS, NP, EOH and AP was used to identify each lipid (sub)class. Ceramides are named according to the nomenclature defined by Motta et al. 16 and extended by Masukawa et al. 17. Briefly, sphingosine (S), dihydrosphingosine (dS), phytosphingosine (P) or 6-hydroxysphingosine (H) represent the sphingoid base structures to which an esterified ω-hydroxy (EO), α-hydroxy (A) or non-hydroxy (N) fatty acid specie is attached via an amide bond. The SC lipid composition of at least 2 human SC donors and 2 LEMs, each generated with the same modified medium, were examined.

Fourier transform infrared spectroscopy (FTIR)

SC samples for FTIR measurements were hydrated at room temperature for 24 hours over a 27% (w/v) NaBr solution. The hydrated SC samples were measured by FTIR as described before 5. Each spectrum in the frequency range of 600 to
4000 cm\(^{-1}\) was obtained during 4 minutes in transmission mode as a co-addition of 256 scans with a resolution of 1 cm\(^{-1}\) with a Varian 670-IR FTIR (Agilent Technologies, Santa Clara, CA) equipped with a mercury-cadmium-telluride detector. The phase transitions of the SC lateral lipid organization were examined as a function of temperature from 0\(^\circ\)C to 90\(^\circ\)C with a heating rate of 1\(^\circ\)C/4 min. The FTIR spectra were analyzed with Win-IR pro 3.0 from Bio-Rad (Bio-Rad Laboratories, Cambridge, Massachusetts, USA). At least 2 SC sheets of human SC and LEMs generated with a modified medium composition were measured.

**Small angle x-ray diffraction (SAXD)**

SAXD measurements were performed at the European Synchrotron Radiation Facility (ESRF) at station BM26B in Grenoble as described before\(^2,18\). Prior to the measurement, SC samples were hydrated as described above. The SC samples were placed in specially designed holders and measured at room temperature for 10 minutes. The intensity of scattered x-rays was measured as function of q. Repeat distances of lamellar phases are calculated from the peak positions (q-values) as described elsewhere\(^5\). At least 2 samples of human SC and LEMs generated with a modified medium content were measured.

**Free fatty acid analysis and quantification by LC/MS**

Extracted SC lipids were dissolved in chloroform/methanol/heptane (2½:2½:95) to obtain a final lipid concentration of 1 mg/ml. In addition, deuterated stearic acid and deuterated lignoceric acid were added to each sample as internal standards, both with a final concentration of 10 \(\mu\)M. An Alliance 2695 HPLC system (Waters Corp., Milford, MA) was used to inject 10 μl of each sample. A C18 analytical column (LiChroCART Purospher STAR, 55 x 2 mm i.d. Merck, Darmstadt, Germany) was used to separate all free fatty acids using gradient elution at a flow rate of 0.5 ml/min starting from acetonitrile: water (90:10) to methanol: heptane (90:10). In addition, 0.1% acetic acid and 1% chloroform were added to both
mobile phases to obtain chloro adducts, greatly enhancing the ionization efficiency 
19, 20. A TSQ Quantum MS (Thermo Finnigan, San Jose, CA) was used to analyze 
all free fatty acids as [M+Cl]⁻ ions. Using an APCI source, the MS was scanning in 
negative ion mode using a scan range from 200-600 amu while maintaining a 
discharge current of 5 μA. The scan time was set to 500 ms while the resolution at 
full width half maximum was 0.7. The capillary and vaporizer temperature were set 
to respectively 250 and 450ºC. An extensive method description will be reported 
elsewhere (Van Smeden et al., in preparation). Afterwards, data was analyzed and 
quantified using Xcalibur software (version 2.0.7, Thermo Scientific, San Jose, CA).

**RESULTS**

*Human SC fatty acids mainly consists of saturated fatty acids, while the SC of LEM shows an increased presence of mono-unsaturated fatty acids*

The SC fatty acid chain length distribution and saturation of LEM and human skin 
were quantified from LC/MS data to determine to which extent they differ from 
each other. Figure 1 shows the free fatty acid species present in human SC (A) and 
in LEM (B). In order to compare the relative chain length distribution of the fatty 
acids in human SC and LEM, the fatty acids were divided into two groups. The 
long chain fatty acids (LCFAs) are defined as fatty acids with 16-21 carbon atoms, 
while the very long chain fatty acids (VLCFAs) are defined as fatty acids with 22-
38 carbon atoms. In these groups both the saturated fatty acids and MUFAs are 
included. LEM has an almost 1:1 molar distribution of VLCFAs and LCFAs, while 
human SC contains approximately three times more VLCFAs compared to LCFAs 
(figure 1C). The seven most prevailing fatty acid species in human SC and LEM 
are provided in table 2. In human SC lignoceric acid (C24:0) and cerotic acid 
(C26:0) account for more than 50% of the total amount of fatty acids. In LEM, 
however, a drastic reduction in lignoceric and especially cerotic acid (both account 
for approximately 33% of the total fatty acid level) is observed together with a
Figure 1. Three dimensional multi-mass LC/MS chromatogram of FFAs in native human SC (A) and control LEM (B) are shown. The retention time is shown on the X-axis, the mass (in amu) is provided on the Y-axis and the intensity of each peak is depicted on the Z-axis. Figure C: relative molar distribution of long chain fatty acids (LCFAs) and very long chain fatty acids (VLCFAs) in the SC of native human skin, control LEM (LEM) and LEM generated with deuterated palmitic acid (LEM C16:0D31). Figure D: relative molar distribution of saturated fatty acids (SFAs) and MUFAs. D1= donor 1, D2= donor 2.
large increase in stearic (C18:0) and oleic (C18:1) acid. When comparing the MUFA level, LEM contains 21-26% MUFAs (figure 1D), while this is only 4% in native human SC. These results clearly demonstrate that LEMs have a decreased VLCFA content and an increased MUFA content compared to native human SC.

**Substitution of protonated palmitic or arachidic acid with their deuterated counterparts does not alter the SC lipid composition or organization**

It is unknown whether the fatty acids that are supplemented to the culture medium are taken up by keratinocytes and are subsequently incorporated into SC lipids. To investigate this fatty acid uptake, protonated palmitic acid (C16:0), which is part of the standard supplement of the LEM culture medium, was substituted by deuterated palmitic acid (C16:0D31). HPTLC analysis revealed that the SC lipid composition of the LEMs fed with deuterated palmitic acid does not differ from the control (data not shown). Both LEMs show the presence of all SC barrier lipid classes, namely cholesterol, free fatty acids and all ceramides subclasses. The free fatty acid chain length distribution and saturation remains similar compared to the control (figure 1C and D). Additionally, both the controls as well as the LEMs treated with deuterated palmitic acid show the presence of only the long periodicity phase with a repeat distance of approximately 12 nm. Additionally, from around 20°C and higher the lipids form only a hexagonal packing (table 3). This indicates that the lipid composition and organization does not differ between the control and LEMs generated with deuterated palmitic acid.

Deuterated arachidic acid (C20:0D39) was used to determine whether fatty acids with carbon chains longer than that of palmitic acid are taken up from the medium and are incorporated in the SC lipids and thereby increase the fraction of SC fatty acids with longer acyl chains. The addition of deuterated arachidic acid does not affect the SC lateral and lamellar lipid organization (table 3) or SC fatty acid composition (figure 4A-C and table 2) compared to the control.
Table 2. Prevailing free fatty acids in human SC and LEMs

<table>
<thead>
<tr>
<th></th>
<th>C16:1</th>
<th>C16:0</th>
<th>C18:1</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C22:0</th>
<th>C24:0</th>
<th>C25:0</th>
<th>C26:0</th>
<th>C28:0</th>
<th>SUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human skin</td>
<td>1.9</td>
<td>11.5</td>
<td>2.2</td>
<td>8.5</td>
<td>0.7</td>
<td>4.5</td>
<td>34.8</td>
<td>6.0</td>
<td>20.9</td>
<td>4.0</td>
<td>93.1</td>
</tr>
<tr>
<td>Control (D1)</td>
<td>ND</td>
<td>11.2</td>
<td>14.4</td>
<td>16.9</td>
<td>4.0</td>
<td>8.4</td>
<td>24.2</td>
<td>0.2</td>
<td>9.5</td>
<td>1.6</td>
<td>90.5</td>
</tr>
<tr>
<td>Control (D2)</td>
<td>2.7</td>
<td>12.8</td>
<td>10.7</td>
<td>16.1</td>
<td>4.6</td>
<td>9.2</td>
<td>23.4</td>
<td>0.7</td>
<td>9.5</td>
<td>2.0</td>
<td>89.1</td>
</tr>
<tr>
<td>+C20:0D39 (D1)</td>
<td>0.8</td>
<td>9.1</td>
<td>9.4</td>
<td>13.7</td>
<td>3.6</td>
<td>8.9</td>
<td>29.5</td>
<td>0.4</td>
<td>15.7</td>
<td>2.7</td>
<td>93.0</td>
</tr>
<tr>
<td>+C20:0D39 (D2)</td>
<td>2.1</td>
<td>14.6</td>
<td>17.4</td>
<td>16.1</td>
<td>4.1</td>
<td>8.3</td>
<td>19.8</td>
<td>0.4</td>
<td>8.1</td>
<td>1.4</td>
<td>90.3</td>
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<tr>
<td>4x FFA mix (D1)</td>
<td>4.2</td>
<td>18.0</td>
<td>25.2</td>
<td>19.8</td>
<td>2.8</td>
<td>4.8</td>
<td>12.5</td>
<td>0.1</td>
<td>4.3</td>
<td>0.7</td>
<td>88.2</td>
</tr>
<tr>
<td>4x FFA mix (D2)</td>
<td>5.3</td>
<td>17.8</td>
<td>33.9</td>
<td>17.3</td>
<td>2.5</td>
<td>4.1</td>
<td>9.1</td>
<td>0.5</td>
<td>3.1</td>
<td>0.6</td>
<td>88.9</td>
</tr>
<tr>
<td>4x C16:0 (D1)</td>
<td>1.2</td>
<td>11.3</td>
<td>7.5</td>
<td>15.4</td>
<td>3.6</td>
<td>9.0</td>
<td>28.2</td>
<td>0.4</td>
<td>15.2</td>
<td>2.5</td>
<td>93.1</td>
</tr>
<tr>
<td>4x C16:0 (D2)</td>
<td>4.8</td>
<td>17.2</td>
<td>23.3</td>
<td>14.4</td>
<td>2.5</td>
<td>5.7</td>
<td>16.8</td>
<td>0.2</td>
<td>8.0</td>
<td>1.3</td>
<td>89.3</td>
</tr>
<tr>
<td>1/2 insulin (D1)</td>
<td>1.6</td>
<td>16.3</td>
<td>13.8</td>
<td>18.8</td>
<td>3.9</td>
<td>7.7</td>
<td>21.2</td>
<td>0.2</td>
<td>7.9</td>
<td>1.3</td>
<td>91.1</td>
</tr>
<tr>
<td>1/2 insulin (D2)</td>
<td>1.1</td>
<td>12.6</td>
<td>12.8</td>
<td>15.7</td>
<td>3.6</td>
<td>8.3</td>
<td>24.0</td>
<td>0.5</td>
<td>11.1</td>
<td>1.8</td>
<td>90.4</td>
</tr>
<tr>
<td>No insulin (D1)</td>
<td>1.2</td>
<td>14.9</td>
<td>16.6</td>
<td>19.4</td>
<td>4.0</td>
<td>7.7</td>
<td>19.7</td>
<td>0.3</td>
<td>7.1</td>
<td>1.1</td>
<td>90.7</td>
</tr>
<tr>
<td>No insulin (D2)</td>
<td>2.1</td>
<td>20.4</td>
<td>13.9</td>
<td>24.3</td>
<td>3.0</td>
<td>6.0</td>
<td>15.8</td>
<td>0.3</td>
<td>6.7</td>
<td>1.1</td>
<td>91.4</td>
</tr>
</tbody>
</table>

Table 2 shows an overview of the relative prevalence (mole %) of free fatty acid species in human skin and LEMs generated with a different medium composition. The seven most prevailing free fatty acids in each sample (highlighted in bold) were determined to examine shifts in prevailing free fatty acid species between different samples. D1= donor 1, D2= donor 2, ND= not detected.

Table 3. SC lipid properties of LEMs generated with different media supplelemts

<table>
<thead>
<tr>
<th></th>
<th>2\textsuperscript{nd} order LPP (nm)</th>
<th>Hexagonal packing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1</td>
<td>Donor 2</td>
</tr>
<tr>
<td>LEM control</td>
<td>6.0 ± 0.1 (n=8)</td>
<td>23.4 ± 3.8 (n=7)</td>
</tr>
<tr>
<td>LEM C16:0D31</td>
<td>5.9</td>
<td>6.0</td>
</tr>
<tr>
<td>LEM +C20:0D39</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>LEM 4x FFAs</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>LEM 4x C16:0</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>LEM no insulin</td>
<td>5.7</td>
<td>5.9</td>
</tr>
<tr>
<td>LEM 0.5x insulin</td>
<td>5.8</td>
<td>5.7</td>
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</table>
Deuterated palmitic and arachidic acid are incorporated in the SC lipid matrix of LEM

FTIR offers the opportunity to determine whether deuterated palmitic and arachidic acid are incorporated in the SC lipids. The vibrations of the deuterated lipids result in shifted peak positions in the FTIR spectrum compared to the peak positions belonging to the vibrations of their protonated counterparts. The FTIR symmetric CH$_2$ stretching vibration observed around 2850 cm$^{-1}$ provides information about the conformational disordereding. The frequency of the CH$_2$ symmetric stretching vibration indicates whether the lipids are present in a crystalline phase, namely the orthorhombic or hexagonal phase, or in the liquid phase $^{21,22}$. When lipids are arranged in a crystalline packing the symmetric CH$_2$ stretching frequency is below 2850 cm$^{-1}$, but when they are in a liquid packing the conformational disorder increases. This is reflected in a steep increase in symmetric stretching frequency to 2852-2854 cm$^{-1}$. In the FTIR spectrum the symmetric stretching frequency of deuterated lipids is located around 2090 cm$^{-1}$. The transition from a crystalline to liquid phase can be recognized by a steep increase in wavenumber to 2096 cm$^{-1}$. The order-disorder transition temperature observed for the controls and LEMs treated with deuterated palmitic or arachidic acid overlap (figure 2A). The presence of the 2090 cm$^{-1}$ CD$_2$ stretching vibration peak in the spectrum of the LEMs cultured with deuterated palmitic or arachidic acid clearly demonstrates that the deuterated fatty acids added to the culture medium are taken up by basal keratinocytes and are present in the SC lipids. To determine whether the deuterated lipids were incorporated into the SC lipid phases or formed separate lipid domains, the CD$_2$ scissoring vibrations around 1090 cm$^{-1}$ were examined. If deuterated palmitic or arachidic acid form separate lipid domains an orthorhombic lateral packing is observed, which is characterized by two vibrations. However, only one peak is observed in the FTIR spectrum of the LEMs treated with deuterated palmitic or arachidic acid (figure 2B). Therefore there are no indications
that separate domains of palmitic or arachidic acid are present in the SC of the LEMs. The deuterated symmetric stretching vibration around 2090 cm$^{-1}$ and scissoring vibration around 1090 cm$^{-1}$ of LEMs treated with deuterated arachidic acid have a lower intensity than observed for LEMs treated with deuterated palmitic acid. This indicates that a higher amount of deuterated palmitic acid is present in the SC of LEM, even though a higher molar amount of deuterated arachidic acid is added to the medium. Taking this into consideration, it is of more interest to supplement the medium with palmitic acid than arachidic acid.

**Figure 2.** A: protonated and deuterated symmetric stretching frequencies around 2850 and 2090 cm$^{-1}$, respectively. B: deuterated scissoring vibration in the control LEM and LEM treated with deuterated palmitic or arachidic acid. The arrow head indicates the deuterated scissoring vibration at 1088 cm$^{-1}$.

**Deuterated palmitic and arachidic acid are present as various elongated fatty acid species in the SC of LEM**

The SC fatty acid composition of LEMs treated with deuterated palmitic or arachidic acid was studied by LC/MS to verify whether deuterated lipids are indeed present in the SC. As can be observed from figure 3, the LC/MS method is able to detect deuterium labelled fatty acids since the added internal standard (C24:0D47) is observed in the chromatograms, bearing a mass of 47.3 amu higher than protonated lignoceric acid (C24:0). The LC/MS profile of LEMs treated with
deuterated palmitic acid show the presence of lipid peaks (figure 3B) which are not observed in the control LEM (figure 3A). The molecular weight of these lipids corresponds exactly to the molecular weight of saturated fatty acid species with a chain length varying from C20:0 to C28:0 with the addition of 31.2 mass units. The increase in 31.2 mass units corresponds to the higher molecular weight of deuterated palmitic acid compared to protonated palmitic acid. These results demonstrate that palmitic acid added to the culture medium is taken up by the keratinocytes and is subsequently elongated in the viable epidermis to longer fatty acids.

Figure 3. Three dimensional multi mass chromatogram and retention times of individual fatty acids in the SC of control LEM (A) and LEM generated with deuterated palmitic (B) or arachidic acid (C). The deuterated fatty acids have a slightly shifted retention time compared to the corresponding protonated fatty acids, similarly as observed for the deuterated internal standard C24:0D47 (IS) and protonated C24:0.
acid species. At the final differentiation step it is used to generate the extracellular lipid matrix in the SC. The LC/MS fatty acid profile of LEMs treated with deuterated arachidic acid also shows the presence of lipid peaks which are not present in the control (figure 3C and A, respectively). These lipid peaks have an increase of 39.2 mass units compared to protonated fatty acids (ranging from C20:0 to C28:0), which corresponds to the molecular weight difference between deuterated and protonated arachidic acid. This indicates that arachidic acid can also be elongated to longer fatty acid species in LEM.

*High amounts of polyunsaturated fatty acids added to the culture medium result in a deteriorated SC lipid organization and free fatty acid composition*

After having demonstrated that palmitic acid in the culture medium can be elongated and incorporated in the SC lipids of LEMs, different approaches were used to increase the SC fatty acid content and to shift their composition in the direction of the VLCFA level observed for human SC. The total fatty acid mixture (palmitic acid, arachidonic acid and linoleic acid; FFA mix) concentration in the culture medium was increased four times. Since we observed elongation of supplemented deuterated palmitic acid to fatty acids with longer carbon chains in the viable epidermis of LEMs, the addition of extra palmitic acid may not only increase the total fatty acid content, but also increase the content of VLCFAs.

HPTLC analysis reveals that the intensity of cholesterol and free fatty acid bands are similar in the control and one LEM donor generated with a fourfold increased FFA mix, while the other donor even showed a reduced free fatty acid intensity compared to the control (figure 4A). This indicates that there is no increase in fatty acid content in SC of LEM when the fatty acid mixture is increased four times. FTIR and SAXD analyses, however, show that the SC lipid organization is altered, albeit not in the desired manner. A fourfold increase in FFA mix supplementation reduces the repeat distance of the lipid lamellae and the temperature at which only a hexagonal phase is present (table 3). The LC/MS results demonstrate that the
increased addition of the FFA mix markedly reduces the level of VLCFAs and increases the MUFA content (figure 4B and C). The main differences in fatty acid composition between the LEMs treated with a fourfold higher FFA mix and the control is an increase in oleic acid and a decrease in lignoceric acid (table 2). Although the palmitic acid concentration is increased in the culture medium, no major increase in palmitic acid level is observed compared to the control (table 2). This study demonstrates that the VLCFA and MUFA content in LEM even further deviates from the VLCFA and MUFA content of human SC when supplementation of linoleic, arachidonic acid and palmitic acid is increased.

Figure 4. A: SC cholesterol (CHOL) and free fatty acid (FFA) content of control LEM (lane 1), LEM with deuterated arachidic acid (lane 2), LEM with a fourfold higher fatty acid mixture (lane 3) and LEM with a fourfold higher palmitic acid content (lane 4). D: SC cholesterol and free fatty acid content of control LEM (lane 1), LEM with a twofold reduction in medium insulin concentration (lane 2) and LEM with medium containing no supplemented insulin (lane 3). B and E: relative molar distribution of long chain fatty acids (LCFAs) and very long chain fatty acids (VLCFAs). C and F: relative molar distribution of saturated fatty acids (SFAs) and mono-unsaturated fatty acids (MUFAs).
Increase of palmitic acid supplementation does not alter SC lipid composition and organization in LEM

Another approach to increase the SC fatty acid content of LEMs was to increase the palmitic acid concentration four times, while the arachidonic (C20:4) and linoleic acid (C18:2) concentration remained similar. These LEMs show no differences in intensity of the fatty acid and cholesterol bands compared to the control in their HPTLC lipid profile (figure 4A). This indicates that the increase in palmitic acid concentration does not lead to dramatic differences in the total free fatty acid level in SC. The increase in palmitic acid concentration also did not affect the lamellar lipid organization or lead to a pronounced difference in the temperature at which only the hexagonal phase is present compared to the controls (table 3). LEMs treated with a fourfold increase in palmitic acid concentration also show no considerable difference in their VLCFA and MUFA content compared to the controls (figure 4B and C). Furthermore, they do not show marked differences in the relative content of individual fatty acid species, including palmitic acid (table 2), compared to the controls. This demonstrates that no pronounced differences in the fatty acid chain length distribution or degree of saturation occurs by only increasing the palmitic acid concentration in the medium four times.

The fourfold increased FFA mix has four times more arachidonic and linoleic acid compared to the four fold increase in palmitic acid supplementation. This indicates that the increased addition of these two polyunsaturated fatty acids to the medium is probably the cause of the deteriorated lipid organization observed in LEMs treated with a fourfold higher FFA mix.
Reduction of insulin concentration in the medium does not reduce SCD1 expression

It has been reported that a reduction in insulin level leads to decreased SCD activity\textsuperscript{10, 11} and thus a reduction in MUFA levels. For this reason the insulin concentration of the medium was lowered. No difference in free fatty acid content can be detected with HPTLC (figure 4D) regardless of the insulin concentration in the medium. Additionally, reduction of insulin supplementation does not lead to differences in lipid organization (table 3), fatty acid chain length distribution or a decrease in MUFA level compared to the control (figure 4E and F). Concurrently, expression of SCD1 in LEMs generated with media containing a reduced insulin concentration remains unaltered compared to the control (figure 5A). Under these conditions SCD1 is expressed in the basal and differentiated layers in LEM, while in human skin the expression of SCD1 is strictly localized in the basal layer.

Peroxisome proliferator-activated receptors (PPARs) are important in the regulation of lipid homeostasis in human keratinocytes. Endogenous fatty acids and their derivatives are activators of PPARs\textsuperscript{23-25}. Forman \textit{et al.}\textsuperscript{26} demonstrated that polyunsaturated fatty acids such as arachidonic and linoleic acid are efficient activators of two PPAR isoforms, namely PPAR\textgreek{a} and PPAR\textgreek{b}/\textgreek{d}. The expression pattern of these two PPARs were therefore determined in LEM (control) and compared to native human skin. LEM shows a similar expression pattern of PPAR\textgreek{a} and PPAR\textgreek{b}/\textgreek{d} in the viable epidermis as native human skin (figure 5B). PPAR\textgreek{a} is mainly expressed in the nuclei of the keratinocytes in the viable epidermis, but also shows some staining of the cytosol. PPAR\textgreek{b}/\textgreek{d} is detected in the nuclei and cytosol of the entire viable epidermis. Altering the free fatty acid supplementation or reducing the insulin concentration in the medium did not result in changes in PPAR\textgreek{a} and PPAR\textgreek{b}/\textgreek{d} expression in LEM (data not shown).
Figure 5. A: Immunohistochemical staining showing the expression pattern of stearoyl-CoA desaturase 1 in native human skin (1), control LEM (2), LEM generated in medium with a twofold lower insulin concentration (3) and LEM generated without insulin in the culture medium (4). B: Immunohistochemical staining showing the expression pattern of PPARα and PPARβ/δ in native human skin and control LEM. Scale bars represent 50 μm.

DISCUSSION

Reconstructed epidermal models mimic epidermal morphogenesis of human skin to a high degree. For this reason a number of commercial HSEs have been used for safety testing of compounds. However, these models do not fully mimic the SC barrier properties of human skin and therefore can provide an overestimation of compound penetration. Only a few studies have been dedicated to optimize the SC barrier properties of HSEs. Generally the culture medium is modulated by supplementing compounds that have beneficial effects on the SC lipid properties of HSEs. This indicates that optimization of the culture medium shows potential to improve the SC barrier properties of HSEs. We demonstrated that the SC of LEM, our in-house HSE, has a decreased free fatty acid level and an increased presence of MUFAs compared to human SC. These two factors may be key factors for the decreased permeability barrier observed for LEM. We aimed to improve the SC fatty acid composition of LEM by adding fatty acids to the culture medium or reducing the medium insulin level. A number of SC barrier properties were examined in great detail by different techniques.
Additionally, a novel LC/MS method was used to quantitatively determine the MUFA level and distribution of FFA species in the SC of LEM. The presented results show that LEM has approximately 1.5x less VLCFAs and 5-6x more MUFAs than observed in human SC. The detection of elongated deuterated fatty acid species in the SC of LEMs after supplementation of the medium with deuterated palmitic or arachidic acid explicitly demonstrates that SC lipids do not necessarily need to be derived from \textit{de novo} synthesis, but may also originate from external sources. Increasing the amount of fatty acids added to the culture medium therefore appeared to be a straightforward approach to increase the SC fatty acid level in LEM. Increasing the palmitic acid supplementation four times, however, does not result in an improved chain length distribution of free fatty acids in LEM. This indicates that the altered free fatty acid distribution observed in LEM compared to human SC is not caused by a deficiency of palmitic acid in the culture medium.

Palmitic acid is a building block for many lipid species, such as longer fatty acids, phospholipids, ceramides, diacylglycerides (DAG) and triacylglycerides (TAG). It is possible that the extra palmitic acid added to the culture medium is incorporated into the latter two lipid species, considering that LEMs have an increased DAG and TAG content (data not shown). Fatty acids may therefore be available to a lesser extent for the synthesis of phospholipids, which are degraded to fatty acids at the stratum granulosum/SC interface\textsuperscript{38}.

LEMs generated without fatty acids supplemented to the culture medium did not show reproducible results concerning their morphology, SC lipid organization and composition. This indicates that fatty acid supplementation is required to generate LEMs. The irreproducible results may be due to keratinocyte donor variations in fatty acid synthesis or differentiation and proliferation responses when free fatty acids are omitted from the culture medium.

LEM has a reduced VLCFA content compared to native human SC. Increasing the VLCFA content in the SC of LEM cannot be achieved by supplementation of the
medium with fatty acids longer than palmitic acid, since they are taken up to a lesser extent by keratinocytes than palmitic acid. In native human epidermis VLCFAs are generated by the activity of several elongases \cite{39, 40}. The reduction of VLCFAs in LEMs may therefore be caused by a reduced activity of elongases.

The deteriorated SC lipid organization of LEMs in which the FFA mix supplementation was increased four times is most probably due to the increased MUFA level. As far as the increased hexagonal packing is concerned, we observed that an increased level of MUFAs in lipid mixtures comprised of fatty acids, ceramides and cholesterol increases the formation of the hexagonal packing (Janssens et al., unpublished data). By increasing the supplementation of the FFA mix four times, the endogenous levels of palmitic, linoleic and arachidonic acid in the keratinocytes may have increased. This may have led to an imbalanced activation of PPARs and consequently an altered SC lipid composition and organization in LEMs treated with a fourfold higher FFA mix.

Insulin is a potent hormone that regulates carbohydrate and fat metabolism in the body. However, the reduction or complete omission of insulin supplementation to the culture medium does not lead to apparent changes in the SC lipid organization or fatty acid composition. It still remains to be established why the LEMs generated with lower insulin concentrations compared to the control show only minor changes in the SC lipid composition and organization. However, several studies have shown that the accumulation of e.g. DAG decrease insulin action \cite{41, 42}. Since LEM have an increased DAG content in the SC compared to human SC, it may indicate that the keratinocytes may have a reduced response to insulin.

Novel approaches are required to improve the SC lipid properties of LEM. SCD1 activity in HSEs needs to be determined and compared to native human skin. If needed, a reduction of SCD1 activity to decrease the MUFA content may be achieved by adding (synthetic) SCD inhibitors \cite{10, 11, 43} to the culture medium.

LEMs show a relatively higher content of DAG and TAG in their SC compared to human SC. DAG and TAG are generated through the action of monoacylglycerol
acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT), respectively. An increase in total fatty acid content may be achieved by partial inhibition of MGAT and DGAT activity.

In conclusion, we have clearly shown that modification of lipid supplementation is not sufficient to improve the SC lipid composition and consequently the SC barrier function of LEM. Therefore, different approaches are needed to optimize the SC lipid composition of LEM. The increased content of MUFAs, DAG and TAG in SC of LEM suggest that the SC lipid composition might be improved by modulating the activity of enzymes such as SCD1, MGAT and DGAT. This may contribute to the development of a new generation of HSEs that harbor a competent SC barrier that even more closely resembles the SC lipid composition and organization of native human SC than the current LEMs.

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