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Lamellar lipid organization and ceramide composition in the stratum corneum of patients with atopic eczema

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The existence of an impaired skin barrier function in atopic eczema (AE, also referred to as atopic dermatitis) has been demonstrated previously, revealing the importance of the skin barrier in the pathophysiology of AE. Loss-of-function mutations in the filaggrin gene (FLG) are known to be a major risk factor for developing AE. However, recent findings report a reduced skin barrier function irrespective of FLG genotype, suggesting the importance of additional factors for an impaired skin barrier in AE.

The skin barrier is primarily provided by the stratum corneum (SC), consisting of enucleated cells surrounded by lipid regions. SC lipids (primarily ceramides (CERs), cholesterol and free fatty acids) form two lamellar phases: the short- and long periodicity phase (SPP and LPP, respectively) with periodicities of ~6 nm and ~13 nm respectively. These periodicities were determined by small angle X-ray diffraction (SAXD). Barrier properties of the skin may also depend on the lipid organization of the SC lipids and an altered lipid composition or organization may cause a reduced skin barrier in AE. Several studies report on the CER composition in AE patients. In the present study, the CER composition and the lamellar lipid organization in SC of AE skin have been simultaneously examined. For AE patients, we observe drastic changes in lipid organization which correlate with changes in CER composition, as compared to control subjects.
The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the Leiden University Medical Center. Subjects gave written informed consent. 6 Caucasian AE patients (23.3±5.2 years; 2 males) and 6 Caucasian subjects without (history of) dermatological disorders (24.7±7.6 years; 1 male) were included. The subjects did not apply any dermatological products to their forearms for at least one week prior to the studies. To study the lipid properties irrespective of FLG mutations, we excluded subjects with any of the four most prevalent mutations found in European Caucasians: 2282del4, R501X, S3247X and R2447X, analyzed by genotyping6.

The lamellar lipid organization was studied using 4 mm biopsies harvested from the ventral forearm of non-lesional skin. Local severity of non-lesional AE skin was evaluated by local SCORing Atopic Dermatitis (SCORAD)7 and was 0 for all patients. SC was isolated by trypsin digestion8 and analyzed by SAXD using a microfocus beam (European Synchrotron Radiation Facility, Grenoble, France) similarly as described elsewhere9. The CER composition was examined in SC harvested by tape stripping non-lesional regions from the ventral forearm (PPS tape, Nichiban, Tokyo, Japan) close to the region of the biopsy. Tape strip numbers 6 to 9 were extracted, pooled and analyzed by liquid chromatography (LC) (Alliance 2695, Waters Milford, USA) coupled to mass spectrometry (MS) (TSQ-Quantum, Thermo Finnigan, San Jose, USA)10,11. The nomenclature of used CERs is described previously12. Briefly, CERs contain a fatty acid chain (either an esterified ω-hydroxy (EO), α-hydroxy (A) or non-hydroxy (N) fatty acid) linked via an amide to a sphingosine chain (either a sphingosine (S), dihydrosphingosine (dS), phytosphingosine (P) or 6-hydroxysphingosine (H)), resulting in 12 CER subclasses.

Figure 1a shows 3 SC diffraction patterns representative for control subjects. The periodicity of the LPP was obtained from the peak positions3. All control subjects show three LPP peak positions (1st, 2nd and 3rd order) at scattering vector values (q) 0.50, 1.00 (main peak) and 1.45 nm⁻¹ respectively, corresponding to a periodicity of 12.6 nm. The 1st order peak of the SPP also contributes to the main peak, which is located at a slightly higher q-value. Figure 1b shows diffraction patterns of AE patients. The top diffraction pattern closely resembles those of control subjects and is representative for 4 patients. However, two patients (marked ⊙ and ◊) show a different pattern: the central curve shows a shift in the main diffraction peak to a q-value of 1.06 nm⁻¹ (labeled *). The lower curve shows only a single reflection at q=1.20 nm⁻¹ (labeled **). Increased q-values of the peak positions signify shorter periodicities of the lamellar phases in SC of those patients. The boxplots (Figure 1c) show a small variance between the 6 control subjects, but a large variance within the group of patients.
Figure 1. Diffraction patterns of (a) SC of control subjects, (b) non-lesional SC of AE patients and (c) boxplots showing the variance of the main peak position in both groups. The inset in the upper right corner of (a) schematically represents the lipid organization and shows the periodicity of the LPP (d=12.6 nm).  and  indicate patients with an altered SAXD profile (indicated by * and **). # Indicates phase separated cholesterol. From the positions of the peaks (q) the repeat distance of the LPP was calculated using the equation 

\[ d = \frac{n \cdot 2\pi}{q_n} \]

(n=order of diffraction peak). The main peak is also caused by the 1st order diffraction peak of the SPP located at a slightly higher q-values than the 2nd order of the LPP.

Figure 2a shows the CER composition analyzed by LC-MS. All 12 CER subclasses were observed, including the newly identified CER [EODs][11]. The observed CER profile is very similar to that observed in previous studies using a fully quantitative method[4,13]. AE skin showed a significant decrease in the content of CER [NP] (P<0.005, student’s t-test) and in the content of long chain CERs (i.e. subclass [EO], Figure 2b, P<0.05, two-way ANOVA), which is in accordance to recent findings[4]. The highest reduction in abundance of CER [EO]-subclasses was observed in patients showing an altered X-ray diffraction pattern:  and in particular  (Figures 1 and 2). Previous studies have shown that the LPP may be important for the skin barrier function[14]: isolated human CERs with a decreased CER [EO] level showed a strong reduction in the formation of the LPP[15]. This variation in structure results in a more dominant influence of the 1st order SAXD peak of the SPP on the main SAXD peak, which explains the shift in peak position in the diffraction patterns of the two AE patients.

To our knowledge, our studies demonstrate for the first time that a change in CER composition can be associated with a change in the lamellar lipid organization in AE patients compared to control subjects. This association may lead to a better
understanding of the altered SC lipid barrier in AE.

**Figure 2.** LC-MS analysis on human SC CERs obtained from tape strips taken from the ventral forearm. To permit semi-quantitative analysis, two deuterated CERs (CER [E(18:2)O(30)S(18)] and CER [N(24)S(18)]) were used as internal standards. (a) Bar graph of the relative CER abundance (in percentages) of every CER subclass in SC of control subjects (green bars) and non-lesional SC of AE patients (maroon bars) (mean±SD, n=12). An unpaired student’s t-test showed a significant difference in NP content between control and AE subjects (P<0.005). (b) Boxplots of the relative CER abundance (in percentages) of the CER [EO] subclasses, as well showing the individual data points (n=12). Green and maroon boxes represent SC of control subjects and non-lesional SC of AE patients, respectively. ANOVA showed a significant difference in the total [EO] content ([EOdS]+[EOS]+[EOP]+[EOH]): P<0.05. Individual AE patients marked as ① and ② correspond to the same patients labeled in Figure 1.

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