The Lipid Organisation of the Skin Barrier: Liquid and Crystalline Domains Coexist in Lamellar Phases

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Abstract. The superficial layer of the skin, the stratum corneum, is the main barrier for diffusion of substances across the skin. The stratum corneum is composed of corneocytes embedded in lipid lamellae. In previous studies two lamellar phases have been identified with periodicities of 6.4 and 13.4 nm of which the 13.4 nm phase (long periodicity phase = LPP) is considered to be very important for the skin barrier function. The main lipid classes in stratum corneum are ceramides, free fatty acids and cholesterol. Until now 8 subclasses of ceramides are identified in human stratum corneum referred to as ceramide 1 to 8. Studies with mixtures prepared with isolated human ceramides revealed that cholesterol and ceramides are very important for the formation of the lamellar phases. After addition of free fatty acids the lipids are organised in an orthorhombic packing with a small proportion of lipids in a liquid phase. Our most recent results show that the presence of ceramide 1 and the formation of a liquid phase are crucial elements for the formation of the LPP. These observations and the broad-narrow-broad sequence of lipid layers in the LPP led us to propose a molecular model for this phase. This consists of one narrow central lipid layer with fluid domains with on both sides a broad layer with a crystalline structure. This model is referred to as 'the sandwich model'.

Key words: lamellar phases, skin, X-ray diffraction

Introduction

One of the most important functions of the skin is to protect the body against exogenous substances from the environment and to reduce the water evaporation from the skin. The barrier for permeation of substances across the skin resides in the upper layer of the skin, the stratum corneum. The stratum corneum consists of keratin-rich dead cells, corneocytes, surrounded by hydrophobic lipid lamellae, which are aligned approximately parallel to the surface of the corneocytes, see Figure 1. Visualisation studies revealed that permeation of substances across the stratum corneum occurs along the lipid domains between the corneocytes [1]. This

Figure 1. A schematic drawing of a skin cross-section. The skin is composed of a dermis and an epidermis. The epidermis is a dynamic, constantly self-renewing tissue. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum undergo a number of changes in both structure and composition. The last sequences of the keratinocyte differentiation are associated with profound changes in their structure, which result in their transformation into chemically and physically resistant cornified squames of the stratum corneum, called corneocytes. In stratum corneum the corneocytes are embedded in lipid lamellar regions, which are oriented parallelly to the corneocyte surface. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions. C = corneocyte filled with keratin. Bar = 100 nm.

implies that the stratum corneum lipid organisation plays a key role in the skin barrier function.

In SC mainly hydrophobic lipids, such as ceramides (CER), long chain free fatty acids (FFA) (most abundant chain lengths C22 and C24) and cholesterol are present. Eight subclasses of ceramides have been identified in human stratum corneum. These human ceramides consist of a sphingosine, a phytosphingosine or 6-hydroxysphingosine base to which a nonhydroxy fatty acid or an α -hydroxy fatty acid is chemically linked [2, 3] see Figure 2. The acyl chain length is mainly C24 and C26 [4]. In human stratum corneum two ceramides, CER1 and CER4, have been identified with a linoleic acid chemically bound to the C31/33 hydroxy fatty acid. Another important lipid in SC is cholesterol sulfate. Although it is present in small amounts, cholesterol sulfate is involved in regulation of the desquamation process in the SC [5].

Figure 2. Molecular structure of human ceramides (A) present in stratum corneum. The molecular structure of CER1-linoleate, CER1-oleate and CER1-stearate (B).

Information on the relationship between lipid organisation and composition is of great importance for understanding the skin barrier function. This is demonstrated in diseased skin, in which an impaired barrier function often parallels altered lipid composition and organisation [6, 7]. To examine the role individual lipid classes play in the stratum corneum, mixtures prepared with CHOL, FFA and human CER have been studied by X-ray and electron diffraction techniques.

In this paper first a brief description of the X-ray diffraction and electron diffraction method will be provided, after which the lipid organisation in intact human SC and in lipid mixtures based on isolated human CER will be described.

X-ray Diffraction and Electron Diffraction

SMALL ANGLE X-RAY DIFFRACTION (SAXD)

The scattered intensity is plotted as function of Q , the scattering vector, which is related to scattering angle (Θ) by $Q = 4\pi \sin \Theta / \lambda$. An important parameter to characterise lamellar phases is its periodicity (d). The diffraction pattern of a lamellar phase is characterised by a series of peaks located at the same inter-peak distance. From the position of the peaks the periodicity (d) can be calculated according to Bragg's law: $d = 2\pi n / Q_n$, in which n is the order of the peak and Q_n the position of the n^{th} order peak.

WIDE ANGLE X-RAY DIFFRACTION (WAXD)

One of the parameters determining the stratum corneum barrier properties is the lateral packing of stratum corneum lipids. Only the liquid, hexagonal and orthorhombic sublattices play an important role in stratum corneum lipid organisation. In the liquid packing (high permeability) the intermolecular distance is not very well defined. This results in a WAXD pattern that is characterised by a very broad peak at 0.46 nm. The diffraction pattern of the two-dimensional hexagonal lattice is characterised by a strong reflection at 0.41 nm. In the case of the orthorhombic sublattice (low permeability) two strong reflections can be detected at a spacing of 0.41 and 0.37 nm, respectively.

ELECTRON DIFFRACTION

When using electron diffraction, an area of approximately $1 \mu\text{m}^2$ is exposed to the electron beam in contrast to WAXD during which the exposed area to the X-ray beam is about 1–2 mm². Due to the small cross section of the electron beam, it is therefore possible to expose only one or a few crystals to the electron beam [8]. As a consequence spots are detected in the diffraction plane. The hexagonal packing results in reflections that are separated by an angle of 60° and are located at a spacing of 0.41 nm. In the pattern of the orthorhombic packing the angle between the reflections is not equal to 60°. Two reflections are located at a spacing of 0.37 nm, while the remaining 4 reflections are located at 0.41 nm spacing [9].

Human Stratum Corneum

The SAXD pattern of human stratum corneum is given in Figure 3. The diffraction curve is characterised by a strong and a weak doublet. Both doublets have a shoulder on the right-hand side (see arrows). Since the number of peaks is limited, and the peaks are very broad and partly overlap each other, interpretation of such diffraction pattern is quite complicated. However, when the diffraction pattern is also measured after recrystallisation of the lipids from 120 °C to room temperature and the peak positions prior and after recrystallisation are compared, it appears that at room temperature two lamellar phases are present in human stratum corneum. One lamellar phase has a periodicity of approximately 6.4 nm, and the other a periodicity of approximately 13.4 nm, respectively [10]. Since the 13 nm lamellar phase has always been present in all species studied so far, this phase is considered to be important for the skin barrier function.

In the WAXD pattern of human SC two rings can be distinguished at 0.37 and 0.42 nm spacing [9], see Figure 4. These rings are attributed to the orthorhombic lateral packing. However, whether a hexagonal sublattice coexists with an orthorhombic sublattice cannot be deduced from the diffraction pattern, since the 0.41 nm reflection characteristic for the hexagonal lateral packing is obscured by the 0.41 nm reflection attributed to the orthorhombic phase. For this reason the lateral

Figure 3. The small angle X-ray diffraction profile of human SC at room temperature and after recrystallisation from 120 °C. The intensity is plotted as function of the scattering vector Q . The high intensity at low angle is due to keratin present in the corneocytes. At room temperature the lipid organisation is characterized by two lamellar phases with periodicities of 6.4 and 13.4 nm. After recrystallisation only the 13.4 nm phase is present. The orders indicated by 2, 3, 4, 5 and 6 are attributed to the 13.4 nm phase. The peaks indicated by I and II are attributed to the 6.4 nm phase.

packing has been recently studied by electron diffraction using sequential strips of SC [8]. With this approach we have established that throughout the SC the lipids form an orthorhombic lattice with the exception of the most superficial layers in which occasionally hexagonal packing has been observed.

CHOL: CER: FFA MIXTURES

In order to study the role various lipid classes play in the lipid organisation, CER were isolated from *human stratum corneum*. In this paper we will only summarize

Figure 4. Lateral lipid packing in human stratum corneum. WAXD pattern of human stratum corneum oriented parallel to the primary X-ray beam. The diffraction pattern is characterized by two rings (0.378 nm and 0.417 nm) indicating that the lipids are organized in an orthorhombic lateral packing. The rings are stronger in equatorial position indicating that the lipids are oriented mainly perpendicularly to the stratum corneum surface. Furthermore, a number of reflections can be attributed to phase separated CHOL. The position of the reflection in the pattern indicates that CHOL crystals have a preferred orientation in a similar direction as the lipid lamellae. The strong broad reflections at 0.46 and 0.92 nm can be attributed to soft keratin.

the phase behaviour of equimolar CHOL:CER:FFA mixtures observed at pH 5, which approximates the pH at the skin surface.

Lamellar phases. In equimolar mixtures prepared from CHOL and CER the major fraction of lipids forms a lamellar phase with periodicities of 12.8 nm [11]. Only a very small fraction of lipids forms the short periodicity phase with a spacing of

Figure 5. The small angle patterns of the equimolar CHOL:CER (1:1) and CHOL:CER:FFA (1:1:1) mixture. 1, 2, 3, 4, 6, 7 and 9th denotes the 1st, 2nd, 3rd, 4th, 6th, 7th and 9th order peak of the pattern based on the LPP. I, II and III refer to the 1st, 2nd and 3rd order of the SPP. * peaks denoted to phase separated CHOL.

approximately 5.5 nm. Variation of the CHOL:CER molar ratio between 0.2 and 1.0 does not change the lipid phase behaviour strongly suggesting that the phase behaviour is insensitive to the CHOL:CER molar ratio. This has also been observed in lipid mixtures prepared from pig ceramides [12]. Addition of FFA promotes the presence of the short periodicity phase while the peaks attributed to the long periodicity phase weakened (see Figure 5).

Lateral packing. In the absence of FFA, a diffraction pattern indicating a hexagonal lateral packing has been observed. The lateral packing is insensitive towards changes in CHOL:CER ratio, similarly as observed for the formation of lamellar phases. Addition of FFA induces a transition from a hexagonal to an orthorhombic lateral packing and the presence of a liquid phase is clearly detected, see Figure 6 [11].

Figure 6. The wide angle patterns of the equimolar CHOL:CER and CHOL:CER:FFA mixtures. * = peaks attributed to phase separated CHOL. Numbers represent the spacings corresponding to the peak positions. Orth = peak attributed to the orthorhombic phase. HEX = peak attributed to the hexagonal phase. 1:1 = curve obtained from equimolar CHOL:CER mixture; 1:1:1 = curve obtained from equimolar CHOL:CER:FFA mixture. Spacings of the orthorhombic and hexagonal lattices are indicated in the figure.

The formation of the orthorhombic lateral packing demonstrates the important role of FFA for formation of a competent barrier.

CHOL:CER MIXTURES PREPARED IN THE ABSENCE OF CER1

When CER1 is absent, as in CHOL:CER(2–8) mixtures, the phase behaviour changes dramatically [11], namely only a very small population of lipids forms the 12.6 nm lamellar phase.

CHOL: CER: FFA Mixtures in Which Natural CER1 is Substituted By Either Synthetic Ceramide 1 Stearate, Synthetic Ceramide 1 Oleate or Synthetic Ceramide 1 Linoleate

To elucidate the role of fatty acid linked to ω -hydroxy acid, natural CER1 was replaced by either *synthetic* CER1-linoleate (CER1-lin), by *synthetic* CER1-oleate (CER1-ol) or by *synthetic* CER1-stearate (CER1-ste), see Figure 2. The following changes in lipid phase behavior were noticed [13]. i) No liquid phase could be detected when HCER was substituted for CER1-ste. ii) Substitution of HCER1 by either CER1-ol or CER1-lin revealed a clearly presence of the liquid phase, being less prominent in mixtures with CER1-lin. iii) As far as the lamellar phases are concerned, the long periodicity phase was not present in HCER:CHOL mixtures in which HCER1 was substituted for CER1-ste. iv) The 12–13 nm lamellar phase was dominantly present in mixtures in which HCER1 is substituted by either HCER1-lin or HCER1-ol. In conclusion, the liquid phase as well as the long periodicity phase were not present when substituting natural CER1 by CER1-ste. *This observation indicates that for the formation of the long peirodicity phase a certain (optimal) fraction of lipids has to form a liquid phase.* More evidence for this relationship has been provided elsewhere [13].

Molecular Organisation in the Repeating Unit of the 12–13 nm Lamellar Phase

The presence of a broad-narrow-broad sequence of the electron lucent bands of the lamellae in the RuO₄-fixed SC [14] and the electron density distribution profile in the repeating unit of the long periodicity phase (calculated from peak intensities by fourier transformations) demonstrating a broad-narrow-broad sequence of the hydrocarbon regions [15] indicate an exceptional molecular organisation in the repeating unit of the 12–13 nm phase. Both observations indicate that the repeating unit consists of 3 layers not equal in width. Based on this and the key role of CER1 in the formation of the 12–13 nm lamellar phase led us propose a model for molecular organisation of the 13 nm lamellar phase, as depicted in Figure 7. In this model the lipids are organised in 3 layers: a narrow layer that is located in the center with a broad layer on both sides. CER1 and CER4 link this tri-layer unit together and consequently dictate the broad-narrow-broad sequence in the tri-layer unit [13]. Due to this lipid arrangement, one would expect a strong cohesion within the tri-layer unit. Imperfections of the lamellar stacking should therefore occur only between different tri-layers but not within the tri-layer units. This is in accordance with observations made during examination of the RuO₄ profiles in the SC: imperfections in the lamellar stacking are always located between adjacent tri-layer units and not within the unit [16].

Previous studies report that in intact SC a subpopulation of lipids forms a fluid phase [17]. Recently it has been demonstrated [11] that also in isolated model systems the crystalline (orthorhombic) phase coexists with the fluid phase. We propose that this liquid sublattice is located in domains in the central layer of the 12–13 nm

Figure 7. Based on the electron density profile calculated from the intensity of 10 orders of diffraction peaks attributed to the long periodicity phase (left panel) and on the broad-broad-narrow sequence of the lamellae visualized in a transmission microscope after RuO_4 (right panel) a molecular model of the long periodicity phase has been presented (central panel). This molecular model is in agreement with the molecular structure of ceramide 1 and ceramide 4 with fully interdigitating linoleate chains. Based upon this molecular model the 'sandwich model' has been proposed [13, 18]. The characteristics are: I. The liquid sublattice is located in the central lipid layer of this phase. In this layer mainly unsaturated linoleic acid and cholesterol are present; II. In the sublattice adjacent to the central layer a gradual change in lipid mobility occurs due to the presence of less mobile long saturated hydrocarbon chains; III. Only a small fraction of lipids forms a fluid phase in the SC. Therefore, one can assume that this central lipid layer is not a continuous fluid phase.

phase [13, 18]. In this layer the unsaturated linoleate and cholesterol are present instead of the long saturated hydrocarbon chains that are present in the adjacent layers. The presence of the fluid phase in this layer is also in agreement with our recent finding that substitution of natural CER1 by synthetic CER1-ste results in a disappearance of the fluid phase [13]. We further suppose that the liquid sublattice in this central layer gradually changes into a structure with a crystalline sublattice. The localisation of a fluid phase in the central layer of the long periodicity phase will have no impact on the skin barrier function, since substances passing the SC regions have always to pass the crystalline regions of the lipid lamellae in the direction perpendicular to the basal plane. We propose that the fluid phase will facilitate the deformation of the lipid lamellae in the stratum corneum [19], especially when applying shear stresses. This model is referred to as *the 'sandwich model'*. We also speculate that localisation of a fluid phase in the central layer will facilitate the transport of molecules into and through desmosomes enabling communication between these structures. Especially water might acquire this penetration pathway.

In Vitro-In Vivo Correlation

One of the key questions is to establish whether the findings obtained with mixtures prepared with isolated SC lipids reflect observations made *in vivo*. To answer this question, information on SC lipid organization in normal and diseased skin has to be obtained. For this purpose, the lipid phase behaviour of the SC derived from healthy, dry skin and lamellar ichthyosis skin has been examined. Dry skin was selected, because of its low content of CER1 [20] and lamellar ichthyosis skin, because of its low content of FFA [21].

For this purpose the lipid composition and lipid organisation of a group of volunteers was studied [22]. It appeared that a change or even an absence of the long periodicity phase was in concert with a reduced content of CER1 and CER4. This finding clearly demonstrates that not only *in vitro*, but also *in vivo* a relation exists between the presence of CER1/CER4 and the formation of the 13 nm lamellar phase.

In a previous study [21] strong evidence has been found that in lamellar ichthyosis skin next to a small change in ceramide composition, the amount of FFA is strongly reduced compared to that in healthy SC. Very recently electron diffraction was used to study lipid organisation in lamellar ichthyosis patients [23]. These studies revealed the predominant presence of a hexagonal sublattice coexisting with a small number of crystals forming an orthorhombic sublattice. The observed deviation in lateral packing in lamellar ichthyosis might be due to a decrease in FFA content as indicated by the results of the lipid mixtures. However, it cannot be excluded that in CER and/or FFA a possible change in acyl chain length occurs and this may affect both the lamellar phase organisation and the lateral packing.

Conclusions

In the past decade many studies have been undertaken to obtain more detailed insight in the SC lipid organisation. It has now become clear that the lipids are organized in lamellae, in which crystalline and liquid domains coexist. This makes it a very exceptional lipid organisation. The important role of CER1 in the formation of the long periodicity phase and the transition from a hexagonal to an orthorhombic phase induced by FFA have been observed in mixtures prepared with isolated stratum corneum lipids as well as in intact human SC. This clearly demonstrates that *in vitro* findings can predict *in vivo* situation.

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