Lipid model membrane systems as a tool for unraveling the underlying factors for skin barrier dysfunction

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Summary and Perspectives

SUMMARY

Introduction

The skin protects the body from the invasion of pathogens and other inimical materials in the external environment, as well as preventing indiscriminate water loss from the body. This barrier function of the skin is attributed to its thin outermost layer known as the stratum corneum (SC) [1-3]. The SC comprises of keratin-filled, flattened dead cells referred to as corneocytes, embedded within a lipid matrix. The corneocytes are surrounded by a protein envelope that reduces the substance uptake. Consequently, the intercellular lipid matrix becomes the major pathway for substances permeating through the skin [4-6]. Thus, the composition and organization of the SC lipids are essential for the barrier function.

X-ray diffraction studies have shown that the intercellular lipids are assembled in two co-existing lamellar phases with periodicities of ~13 nm and ~6 nm known as the long and short periodicity phases (LPP and SPP), respectively [7, 8]. The LPP is a trilayer structure that is exclusively present in the SC and is considered to be important for the skin barrier function. Within the lamellae, the majority of the SC lipids preferably adopt a dense orthorhombic lateral packing at skin temperature (30-32 °C), while a portion of the lipids adopts the less dense hexagonal packing [9, 10]. This dense orthorhombic packing plays an important role in the low permeability of the skin barrier [11].

The major lipid classes found in the SC intercellular lipid domains are cholesterol (CHOL), free fatty acids (FFAs), and ceramides (CERs) [12, 13]. These lipids are present in an approximately equimolar ratio. The FFAs are mainly saturated, with their carbon chain lengths ranging between 12 and 30, the most common fatty acid chain length being 22, 24, or 26 carbon atoms [14-16]. The CERs have a diverse chemical structure. They comprise of a sphingoid base (sphingosine, phytosphingosine, 6-hydroxysphingosine, dihydrosphingosine, or dihydroxy dihydrosphingosine) linked to an acyl chain (Non-hydroxy, α-hydroxy or ω-hydroxy) via an amide bond. The CERs also show variation in their acyl chain length. The most abundant non-hydroxy or α-hydroxy acyl chain lengths are 24 and 26 carbon atoms. Four of the CER subclasses often referred to as acylCERs, constitute an ultra-long ω-hydroxy acyl chain, which contains up to 30-34 carbon atoms, ester-linked to an unsaturated fatty acid (usually linoleic acid) and amide-linked to one of the various sphingoid bases. These acylCERs are crucial for the formation of the LPP. At least 18 CER subclasses have been identified in human SC (Figure 1) [17-22].

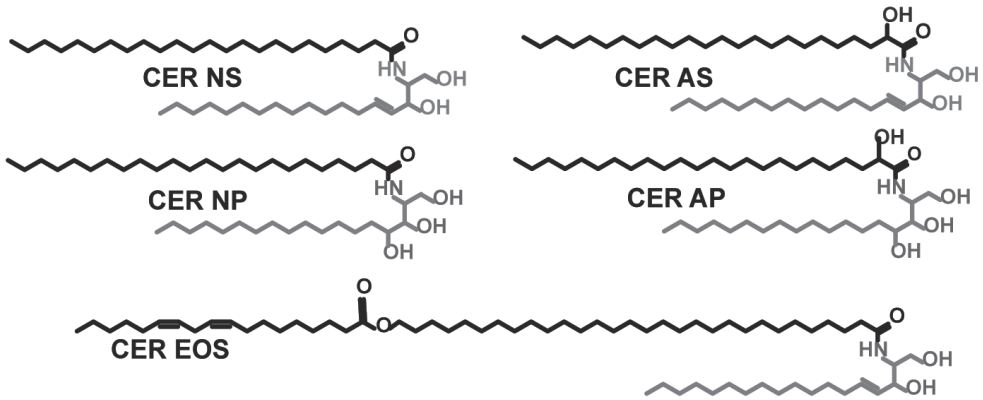

Figure 1: Some CER subclasses in the stratum corneum lipid matrix.

CERs consist of a sphingoid base linked to a FA chain. The acyl chain can either be nonhydroxylated (N), α-hydroxylated (A), or esterified ω-hydroxylated (EO), while the sphingoid base is either sphingosine (S) or phytosphingosine (P).

The composition of the SC lipids is altered in several inflammatory skin diseases including atopic dermatitis (AD), autosomal recessive congenital ichthyosis (ARCI), Netherton syndrome, and psoriasis [23-29]. Common to the skin of the patients of these diseases is barrier function impairment. Consequently, harmful substances gain access to the body. Aside from other factors such as reduced expression of the epidermal barrier proteins, several changes in SC lipid composition occur simultaneously in these patients. Therefore, although correlations between barrier function and change in lipid composition can be found in vivo, it is impossible to determine the effect of alterations in individual lipid classes on the SC lipid organization and skin barrier function in clinical studies. To determine the role of lipids in the impaired barrier properties in inflammatory skin diseases systematically, precluding any other interfering factors, model membrane systems based on synthetic lipids offer an excellent alternative.

The studies described in this thesis aimed to unravel the underlying factors and mechanisms for the impaired skin barrier function in inflammatory skin diseases, particularly focusing on AD, using model membrane systems mimicking the human SC lipid composition and organization.

Development of healthy human skin lipid model

As the composition can easily be modified in model membranes, lipid model

membranes offer the opportunity to mimic the lipid composition and organization in healthy as well as in diseased skin and thus allows for a detailed study of the relationship between lipid composition, molecular organization, and barrier function. In previous studies, the pigCER model comprising of synthetic CERs, CHOL, and FFAs in an equimolar ratio was used as a suitable model for healthy skin (control) to investigate the relationship between the altered lipid composition, lipid organization, and barrier function in psoriasis, Netherton syndrome, and X-linked lamellar ichthyosis skin model. However, the composition of the pigCER model is not fully representative of the CER composition in SC of healthy skin. The pigCER subclass composition is different from that in the human SC. Therefore, in **Chapter 2,** a lipid model membrane mimicking the human SC CER composition and organization was developed. The lipid organization and permeability of the lipid model membrane were compared with those of the human SC to establish the resemblance of the SC model to the native human SC. In subsequent studies, the lipid composition was modulated to mimic several aspects of the lipid composition of SC in AD skin. The changes in lipid composition investigated in this study include i) incorporation of short-chain length CERs with a total chain length of 34 C atoms ii) increased levels of CERs NS and AS, reduction in the level of CER NP iii) reduction in CER EOS concentration and iv) increased level of short-chain FFAs. To determine the effect of each of these alterations on the barrier function two complementary techniques were employed. *In-vitro* permeation study was carried out to measure the amount of a model drug ethyl-p-aminobenzoate (E-PABA) permeating the various membranes per unit area per unit time (flux) (outside-inside barrier), while transepidermal water loss (TEWL) measurements were performed to monitor the water loss across the models (inside-outside barrier). The effect of the various lipid compositional changes of AD skin on the lipid organization was analyzed.

From our result, the healthy skin model membrane properties were very similar to that of the lipid matrix in human SC, exhibiting 2 lamellar phases (SPP and LPP), orthorhombic lateral packing, and showed no significant difference in permeability to E-PABA when compared to the native skin. When the effect of individual changes in the lipid composition of AD skin on the barrier function was evaluated, the largest reduction in barrier function was observed in the model with an increased fraction of short-chain FFAs, attributed to the decrease in chain packing density deduced from the infrared spectrum. X-ray diffraction studies showed that reduction in the concentration of CER EOS resulted in a lower fraction of lipids forming the LPP. Our findings suggest that in the treatment of AD, focusing on the normalization of the FFA composition is at least as important as the normalization of CER composition.

Simple model systems for a detailed evaluation of the relationship between lipid composition, lipid organization, and barrier function

The interpretation of the interaction and lipid phase behaviour in simple systems is more detailed than in complex systems as interference from multiple lipid subclasses does not arise and more detailed information can be obtained using deuterated lipids. Consequently, simpler lipid models with a fewer number of components, prepared as an equimolar mixture of CERs, CHOL, and FFA were employed for a more detailed study of the relationship between changes in CER subclass chain length/composition and skin barrier function based on the changes in CER composition of AD patients' skin investigated in chapter 2.

Chapter 3 describes studies involving varying levels of CER EOS in the SC lipid model. As CER EOS concentration affects the formation of the LPP, simple SC models with gradually increasing levels of CER EOS (10/30/50/70/90) mol% were employed for a detailed investigation of the influence of CER EOS concentration on lamellar organization, LPP unit cell structure, lateral organization, and permeability of the model membrane. The results showed that the permeability of the model did not differ when CER EOS concentration ranged between 10 to 30% but increased significantly at 70% and higher concentrations despite the increased faction of lipids forming an orthorhombic phase. Using CER EOS with an unsaturated deuterated C18 chain, it was determined that the fraction of lipids in a liquid phase increased. Most probably this contributes to the increased permeability with CER EOS concentration, while the surrounding CERs and FFAs remained in a crystalline orthorhombic state. The lamellar organization of the models was analyzed. At 10% CER EOS the model formed both the LPP and SPP similar to the native SC while increasing the concentration of CER EOS to 30% and higher resulted in the disappearance of the SPP. The LPP peak intensity distribution in the diffraction pattern was maintained somewhat up to 70% CER EOS concentration and corresponded to that in the unit cell of a complex SC model and the native SC LPP (2^{nd} order peak more intense than 1^{st} and 3^{rd} order peaks) as described previously [30, 31] indicating similarity in the basic LPP structure. The lipid models used in the studies in chapters 4 and 5 were prepared as an equimolar mixture of CERs, CHOL, and FFA with the CER fraction containing 40% CER EOS. This was necessary to prepare models with lipids assembled in the LPP (hereafter referred to as LPP models) as part of the ongoing studies by our group to characterize the molecular interaction and arrangement of lipids within the trilayer LPP structure which until recently has not received much attention.

In **Chapter 4**, studies are described in which simple LPP models were employed for a more detailed study of the effect of different CER headgroups on

the lipid organization and barrier function of SC model membranes. The focus was on sphingosine-based CERs and phytosphingosine-based CERs as their levels are altered in the SC of AD and psoriasis patients. SC models containing CER EOS 40% and sphingosine-based CERs (CER NS or CER AS) as the CER fraction were compared with their counterparts containing phytosphingosine-based CERs (CER NP or CER AP). The permeability was compared by diffusion studies using E-PABA as a model drug, and the lipid organization was characterized by X-ray diffraction and infrared spectroscopy. Both the sphingosine- and phytosphingosine-based CER models formed the LPP, while the latter exhibited a longer LPP repeat distance and resulted also in additional phases. The E-PABA flux across the sphingosine-based CER models was higher when compared to the phytosphingosine counterparts, while the α-hydroxy phytosphingosine-based CER model had the lowest chain packing density. The unanticipated low permeability of the α-hydroxy phytosphingosine-based model is probably associated with a stronger headgroup hydrogen bonding network. Our findings indicate that the increased level of sphingosine-based CERs at the expense of phytosphingosine-based CERs, as observed in the diseased skin, possibly contribute to the barrier function impairment.

In **Chapter 5**, LPP models with varying levels of short-chain CERs were prepared for a detailed study of the effect of increased concentrations of short-chain CERs observed in SC of AD patients. The level of the long, physiological acyl chain length CER NS(C24) in the SC model membrane was gradually substituted by short-chain CER NS(C16) (0/25/50/75)% in a simple four-component skin lipid model. The effect of short-chain CERs on the permeability and phase behaviour of model membranes in the LPP models was systematically investigated. From our results, the weakest barrier was obtained in the SC model when 75% of the CER NS(24) was substituted with CER NS(C16) resulting in the formation of separate domains, especially of FA(C24) and a change in the LPP structure. This could be deduced from the SAXD and FTIR studies. Relating these findings to diseased skin, the increased level of C34 CERs reported in several inflammatory skin diseases including AD and Netherton syndrome contributes to the impaired barrier function.

CONCLUSION

Using a lipid model that mimics the human CER composition which was systematically altered to mimic various lipid compositional changes observed in

inflammatory skin disease, it was shown that the increased level of short-chain FFAs resulted in a greater reduction in barrier function than changes in the CER subclass/chain length. The former resulted in the reduction in the packing density of the lipid chains while modulations in the CER subclass composition impacted the lamellar organization and head group interactions.

The studies presented in this PhD thesis further show that simple models mimicking important aspects of the native SC lipid organization are suitable for a more detailed study and deeper understanding of the role of lipids in the changes in lipid organization as observed in inflammatory diseases. Thus, unraveling the factors and mechanism underlying the barrier function impairment which is paramount for appropriate targeting and optimization of therapy.

PERSPECTIVES

Further development of the skin lipid membrane model

Although the human skin lipid model presented in this project proved to be an effective tool for studying the effect of the altered lipid composition reported in AD patients' skin on the lipid organization and barrier function, certain improvements can be made for the model to resemble the native skin more closely. Although the CER composition contained the most prevalent CER subclasses, the model was limited as several subclasses were commercially not available. Therefore, it will be of interest to incorporate a wider range of CERs in the model as soon as these CER subclasses become available. The CER composition of the human skin model also differed from the native skin by having an almost uniform chain length: CER EOS ultra-long acyl chain contained 30 carbons, while the acyl chain of all the other CER subclasses contained 24 carbon atoms. Incorporation of a wider chain length distribution of CERs varying in acyl chain as well as sphingoid base chain length as present in the native skin should also be considered as chain length distribution affects the lipid barrier [32].

In addition to the major lipid classes, the SC lipid matrix contains a small amount of CHOL sulphate, which plays a crucial role in the desquamation process. Accumulation of CHOL sulphate was observed in the scale of X-linked ichthyosis patients [33, 34]. The human skin model prepared in this work did not contain CHOL sulphate but was effective for the study of AD since the concentration of CHOL sulphate is not affected in AD. In a previous study of SC models prepared with isolated CERs, CHOL, and FFAs, the lipid phase behavior was not affected

when CHOL sulphate was included in the lipid mixture, except that the solubility of CHOL increased [4, 35]. It would be of interest to examine the influence of CHOL sulphate in membranes based on synthetic CERs. The incorporation of CHOL sulphate 3.4% (of total lipid) in the lipid composition of the human skin model will increase its resemblance to the native skin lipid composition [34, 36] as well as increase its application.

The tortuous intercellular route filled by the lipid matrix, which is the main penetration pathway of substances into the body is not present in the lipid membranes as they do not contain corneocytes. Consequently, the thickness of the lipid membrane barrier is different from that of the SC. Abnormalities in the composition and formation of the bounds lipids linked to the cornified protein envelop surrounding the corneocyte are observed in AD and ARCI patients' skin [27, 37]. This could result in increased permeation through the corneocytes. It would be of interest to construct the tortuous intercellular permeation pathway in the skin model. This may be done by the introduction of synthetic corneocytes. The synthesis of corneocytes has been reported previously. This was done from hyperbranched polyglycerol hydrogel microparticles [38], covered with a monolayer of lipid (ω-hydroxyCER) on the membrane support. Subsequently, the lipid mixture mimicking the composition of the intercellular lipids is applied. By doing so, the permeation route of the native skin in healthy and diseased skin, as well as alterations in composition and structure of the cornified protein and lipid envelope can be mimicked.

Applications of the lipid model membrane

The SC lipid model can be of use for unraveling various factors underlying barrier dysfunction in inflammatory skin disease as well as treatment optimization study. In certain inflammatory skin diseases, the activity of enzymes involved in lipid metabolism is altered resulting in the impaired conversion of lipid precursors into CERs [39]. The activity of sphingomyelinase, which catalyzes the conversion of sphingomyelin to CER NS and CER AS was shown to be reduced in lesional and non-lesional AD skin, correlating with reduced SC CER content and barrier dysfunction. Therefore, it would be of interest to investigate the contribution of the altered sphingomyelinase activity to the reduced barrier in AD and its effect on lipid organization. Human SC models in which the CERs are gradually substituted with their precursor (sphingomyelin) can be used for these analyses. Similarly, the relationship between glucocerebrosidase deficiency observed in Gaucher's disease, SC lipid organization, and epidermal permeability barrier function can be studied using the SC lipid model. For the study, the CER

precursor, glucosylceramide could be incorporated in the skin model mixture at the expense of CERs.

Restoring skin barrier properties by the use of topical formulations aimed to improve the compromised barrier has been targeted in the treatment of skin barrier defects in AD patients [40, 41]. Understanding the action of the topical application, its interaction with the SC lipids, the effect on permeability is important for the preparation of an effective formulation. It will be of interest to study the interaction of the models with sebum, which is a natural moisturizer present on the body surface, comprising of a mixture of glycerides, FFAs, wax esters, squalene, CHOL esters, and CHOL. Skin barrier lipid components aimed to normalize the lipid composition can be systematically incorporated into the sebum lipid mixture resulting in various formulations. The effect of the formulations on the lipid organization and permeability of the model can then be investigated to select those components that normalize the skin barrier.

Due to the multifactorial nature of AD, the causes and severity of the disease can vary between patients. As there is a large variation in the CER and FFA composition of patients, one-size-fits-all treatment for different patients may not be ideal. The human skin model can offer the possibility of personalized medicine for AD patients. This is because individual patients' skin lipid composition can be directly mimicked and analyzed. A personalized treatment plan that will lead to optimal recovery of barrier function could then be established using the models, tailored to the individual patient's circumstance.

The human SC lipid model can be used for screening candidates for use as moisturizers, which are formulations applied for the treatment of dry skin. Their effectiveness in improving the barrier can be assessed. The use of the model membrane offers the opportunity to study the effect of the moisturizer on lipid organization and unravel the mechanism of action. Similarly, candidates for penetration enhancement, which are substances that increase the permeation of the skin can also be evaluated using the human skin model.

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