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Review article

Corneocyte lipid envelope (CLE), the key structure for skin barrier function and ichthyosis pathogenesis

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Abbreviations: ABC, ATP-binding cassette; ABCA12, ATP-binding cassette sub-family A member 12; ABHD5, α/β-hydrolase domain-containing protein 5; ARCI, autosomal recessive congenital ichthyosis; CCE, cornified cell envelope; CERS, ceramide synthase; CIE, congenital ichthyosiform erythroderma; CLE, corneocyte lipid envelope; CYP4F22, cytochrome P450 4F22; ELOVL, elongation of very-long-chain fatty acid; eLOX3, lipoxygenase-3; ER, endoplasmic reticulum; FATP4, fatty acid transport protein 4; LI, lamellar ichthyosis; PNPLA1, patatin-like phospholipase domain-containing protein 1; TGase, transglutaminase; 12R-LOX, 12(R)-lipoxygenase; UGCG, UDP-glucose ceramide glucosyltransferase; ULC, ultra-long-chain

ABSTRACT

Research on the genetic abnormalities and pathogenetic processes of ichthyoses has progressed remarkably, and many causative genes and molecules have been identified in ichthyoses and ichthyosis syndromes. Most of the genes/molecules causative of ichthyosis are associated with the barrier function of the stratum corneum, and defects in the skin barrier play important roles in the pathogenesis of various ichthyosis phenotypes.

It has been elucidated that, of the ichthyosis-causative genes, *ABCA12*, *ALOXE3, ALOX12B*, *CYP4F22, CERS3, ABHD5*, *PNPLA1* and *ELOVL4* work in the formation of the corneocyte lipid envelope (CLE), a structure that is essential to sound skin barrier function. The CLE mostly consists of ultra-long-chain (ULC) ceramides derived from ULC-acylceramide (EOS; a combination of esterified ω-hydroxy fatty acids and sphingosines).

In this review, I shed light on the synthesis, metabolism and transport of epidermal ceramides, especially on ULC-acylceramide and the processes of CLE formation. In addition, I summarize the pathogeneses of various ichthyoses and ichthyosis syndromes from the aspects of abnormal synthesis of ULC-acylceramide and malformation of the CLE.

Investigations on the pathomechanisms of ichthyoses have provided novel knowledge on the synthesis and metabolism of ceramides in the epidermis. Conversely, research on the dynamics of epidermal ceramides has contributed to the elucidation of the pathogenesis of ichthyoses.

Advances in our understanding of the biology of epidermal lipids and the disease pathogeneses of ichthyoses and ichthyosis syndromes promise to provide clues for the development of effective therapies for ichthyosis patients in the near future.

Key Words: acylceramide, ceramide, corneocyte lipid envelope, EOS, ichthyosis syndrome

1. Introduction

The ichthyoses are a group of clinically and etiologically heterogeneous keratinization diseases that generally affect much or all of the skin [1]. Their clinical severity ranges from that of harlequin ichthyosis, the most severe form, to that of ichthyosis vulgaris, the mildest form [2]. For a long time, the pathogenetic mechanisms and causative genetic abnormalities of ichthyoses remained to be clarified. Recently, considerable advances have been achieved in our knowledge of the molecular basis of epidermal keratinization and skin barrier formation [3].

Many disease phenotypes of ichthyoses have primary causes and pathogenetic mechanisms associated with defective barrier function in the stratum corneum [1]. The barrier structure of the stratum corneum in human skin has four major components, from the inside of cornified cells to the outside: keratin/filaggrin and their degradation products filling the cytoplasm of cornified cells, the cornified cell envelope (CCE), the corneocyte lipid envelope (CLE) and the intercellular lipid layers (Fig. 1). Of these structures, the CLE and the intercellular lipid layers consist of essential epidermal lipid components. Ceramides are the most important components of the intercellular lipid layers in the stratum corneum. The CLE is mainly composed of ultra-long-chain (ULC) acylceramide.

Mutations in a number of genes have been identified in ichthyoses [4] and ichthyosis syndromes [5]. Twelve causative genes have been identified in autosomal recessive congenital ichthyosis (ARCI), including in lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE): *TGM1* and *ABCA12* (two lipoxygenase genes), *ALOXE3* and *ALOX12B*, *NIPAL4, CYP4F22, CERS3, LIPN*, *PNPLA1, SLC27A4, SDR9C7;* and *CASP14* [6, 7]. In addition, a number of genes causative of ichthyosis syndromes have been identified. Not a few genes/molecules among them are involved in the synthesis and metabolism of epidermal lipids [8].

The recent identification of causative molecules of ARCI and ichthyosis syndromes has provided clues towards clarifying how ULC-ceramides and the CLE are closely associated with the pathogenesis of ichthyoses [8]. For a long time, little was known about the synthesis, metabolism and transport of epidermal ceramides. However, recently, we have obtained important information on ULC-acylceramide synthesis, metabolism and transport from the newly elucidated pathogenesis of ARCI and ichthyosis syndromes. In this context, the novel understanding of ichthyosis pathogenesis has opened up a new era of epidermal lipid biology. The elucidation of genetic causes of ARCI and ichthyosis syndromes has contributed to our understanding of the synthesis and metabolism of ULC-acylceramides, and advances in epidermal ceramide biology have provided clues towards clarifying the pathomechanisms of ARCI and ichthyosis syndromes.

In the present article, I summarize the recent progress in our knowledge on the pathogenetic mechanisms of ARCI and ichthyosis syndromes, focusing on abnormalities of ULC-acylceramide synthesis/metabolism/transport and CLE formation as important causal factors in skin barrier defects and ichthyosis phenotypes.

2. The CLE is an essential component of the barrier system of the stratum corneum

As described above, four major components of stratum corneum structures are essential for stratum corneum barrier function: 1) keratins, filaggrins and their degradation products in stratum corneum cells, 2) the CCE of the stratum corneum cells, 3) the CLE of the stratum corneum cells and 4) intercellular lipid layers of the stratum corneum (Fig. 1). The extracellular space of cornified cell layers is occupied by multiple lipid layers called “intercellular lipid layers” that consist mainly of

ceramides, cholesterols and free fatty acids. The CLE is a thin, single lipid layer composed of ULC-ceramides and ULC fatty acids that is located between the CCE and the intercellular lipid layers in the stratum corneum [9]. Recently, it was elucidated that the CLE is closely related to the pathogenesis of many types of ichthyoses and ichthyosis syndromes [9]. The CLE is mostly composed of ω-hydroxyceramide with ULC fatty acids. The ω-hydroxyceramide with ULC fatty acids are EOS (a combination of esterified ω-hydroxy fatty acids and sphingosines) derivatives from the fused limiting membrane of lamellar granules and/or a pool of secreted extracellular acyl-ceramides.

The CLE, the single layer of ULC ω-hydroxyceramides and a small amount of ULC fatty acids, is covalently bonded to the outer surface of the CCE and covers the extracellular surface of the CCE.

As the bond between the outer surface of the CCE and the inner surface of the CLE, glutamyl residues of involucrin and other CCE precursor proteins covalently bind to ω-hydroxy residues of ω-hydroxy ULC-ceramides and ω-hydroxy ULC fatty acids. It is hypothesized that this linking is catalyzed by transglutaminase (TGase) 1 [10], although this hypothesis is controversial [11]. The CLE, which is bonded tightly and covalently to the outer surface of the CCE, is an essential structure as a scaffold for intercellular lipid layers, in the stratum corneum.

3. The CLE formation process, and CLE abnormalities causative of ARCI and ichthyosis syndromes

Ichthyosis and ichthyosis syndromes with autosomal recessive inheritance often have severe ichthyosis phenotypes. Recently, whole-exome sequencing by a next-generation sequencer has dramatically contributed to the elucidation of causative genes/molecules of autosomal recessive ichthyosis and ichthyosis syndromes, although there are still ichthyosis cases of unknown etiology.

A large number of reports clearly indicate that aberrant lipid metabolism, transport and/or secretion by keratinocytes lead to the malformation of the CLE and the intercellular lipid layers in the stratum corneum, constituting major causative mechanisms of genetic ichthyosis [8]. A number of molecules causative of ichthyosis have been found to be critically engaged in CLE formation (Table 1).

Recently, detailed processes of ULC-acylceramide biosynthesis and CLE formation have been clarified gradually. There are a number of steps in the formation of CLE, and not a few enzymes are involved in the process (Figs. 2, 3). All the known enzymes involved in acylceramide production in the epidermis are located in the endoplasmic reticulum (ER), and most of the steps in acylceramide production are thought to occur in the ER [12]. Each step is described as follows.

3.1. Elongation of fatty acids

The elongation of fatty acids is a process that is catalyzed by several elongation enzymes. Starting with palmitoyl-CoA, the elongation enzymes add carbon chains derived from malonyl-CoA to the fatty acids. Elongation of very-long-chain fatty acids protein (ELOVL) 6, ELOVL3, ELOVL7, ELOVL1 and ELOVL4 are successively engaged in the elongation processes [8]. ELOVL4 works in the final step of ULC fatty acid CoA formation. Clinically, loss-of-function mutations of ELOVL4 lead to a syndrome of ichthyosis, intellectual disability and spastic quadriplegia [13].

3.2. ω-hydroxylation of ULC fatty acids

The next important step of ULC-acylceramide synthesis for CLE formation in epidermal keratinocytes is hydroxylation of the ULC fatty acid at the ω position. This step is catalyzed by cytochrome P450 4F22 (CYP4F22) [12].

Lefèvre et al. [14] identified *CYP4F22* mutations as a genetic cause underlying lamellar ichthyosis type 3. CYP4F22 is a fatty acid ω-hydroxylase. Recently, Ohno et al. [12] identified CYP4F22 as the long-sought fatty acid ω-hydroxylase working on ω-hydroxylation of ULC fatty acid for acylceramide production. The report demonstrated that CYP4F22 catalyzes the ω-hydroxylation of fatty acids with carbon chain lengths of 28 or more, on the cytoplasmic side of the endoplasmic reticulum. Lipid analysis of a lamellar ichthyosis patient with *CYP4F22* mutations revealed significantly decreased acylceramide production [12].

3.3 CoA addition to ULC fatty acids

Fatty acid transport protein 4 (FATP4), encoded by *SLC27A4*, is a major fatty acid CoA synthase for ULC fatty acid production [15]. By the action of FATP4, ULC fatty acid CoA is synthesized in the epidermis. Reduced ULC fatty acid CoA synthesis activity due to FATP4 mutations causes ichthyosis prematurity syndrome [16].

3.4. Ceramide synthesis

Ceramide synthase (CERS) 3 and CERS4 are the major ceramide synthases in the skin. CERS3 catalyzes the N-acylation of sphingosine with ULC-acyl-CoA (≧C26) in the upper epidermis, resulting in ULC ceramide generation [17]. Defective CERS3 function leads to ARCI [18, 19].

3.5. ω-O-esterification of ceramides with linoleic acid

α/β-hydrolase domain-containing protein 5 (ABHD5), also termed as CGI-58, is a co-factor that is generally present in the tissues of the mammals, including in the epidermis. It activates adipose triglyceride lipase [20] and its expression increases during keratinocyte differentiation [21]. ABHD5 facilitates the lipolysis of triglycerides to provide fatty acids for ω-O-esterification leading to acylceramide formation [22]. Linoleic acid supplied indirectly as a result of the function of ABHD5

is essential for ULC-acylceramide synthesis and CLE formation. Mutations in ABHD5 result in Dorfman-Chanarin syndrome (a neutral lipid storage disease that features ichthyosis) [23].

ULC-acylceramide with linoleic acid is essential for CLE formation. Acylceramides containing ω-O-esterified fatty acids other than linoleic acids cannot be converted to covalently protein-bonded ω-hydroxyceramide, which forms the CLE. Loss-of-function mutations in *PNPLA1* are known to cause ARCI [24]. Very recently, it was revealed that patatin-like phospholipase domain-containing protein 1 (PNPLA1) plays an essential role in the synthesis of linoleoyl ω-O-acylceramide, from analyses of PNPLA1 knockout mice [25, 26] and from cell-based assays and in vitro analyses [27]. It was clearly demonstrated that PNPLA1 catalyses ω-O-esterification with linoleic acid to produce aclyceramide and that triacylglyceride acts as a linoleic acid donor [25, 27].

3.6. Glucosylation of ceramides

As mentioned above, ceramides are synthesized in ER and move to the Golgi apparatus. In the Golgi, UDP-glucose ceramide glucosyltransferase (UGCG) adds UDP-activated glucose to ceramides at the cytosolic surface [28]. An ichthyosis-like phenotype due to impaired keratinocyte differentiation is seen in UGCG-deficient mice [29]. However, there have been no reports of human disease associated with UGCG deficiency.

3.7. Transport of ceramides via lamellar granules

Lamellar granules are lipid-transporting, secretory granules of epidermal keratinocytes. Lamellar granules contain precursors of the CLE and intercellular lipid layers in the stratum corneum, and a series of hydrolytic enzymes [30].

ATP-binding cassette (ABC) sub-family A member 12 (ABCA12) is a member of a large superfamily of ABC transporters that use ATP to transport various molecules

into a vesicle or across a limiting membrane. Members of the ABCA subfamily are considered to be lipid transporters, and ABCA12 is a lipid transporter that is mainly expressed in keratinocytes and that works in the transport of lipids including ceramides into lamellar granules [31]. In this context, ABCA12 is essential for the formation of the CLE and intercellular lipid layers in the stratum corneum. Mutations of ABCA12 lead to the defective accumulation of the lipid into lamellar granules, resulting in the transport failure of lipids, including ceramides [32]. ABCA12 mutations are genetic causes of almost all harlequin ichthyosis cases and of some CIE cases.

ULC-acylglucosylceramide (glucosyl-EOS with linoleic acid coupled to the ω-hydroxyl) is a constituent of the limiting membrane of lamellar granules and its glucose moieties are on the inside of lamellar granules [33, 34]. The limiting membrane of lamellar granules merges with the plasma membrane at the apical surface of the granular layer cells, and lamellar granules expel their contents into the intercellular gap [35, 36]. The fusion of the limiting membrane of lamellar granules and the cell membrane of the granular layer cells is an early stage of CLE formation. ULC-acylglucosylceramide exposes the glucose moieties on the outer surface of the cell membrane of cornified cells. The ULC fatty acid component of ULC-acylglucosylceramide spans the lipid bilayers of the keratinocyte cell membrane, with linoleic acid folded into the lipid bilayer.

3.8. Oxidation of linoleic acid in ceramides

O-linoleoyl-ω-hydroxy-ULC-glucosylceramide is oxygenated in a region- and stereo-specific fashion by the consecutive actions of two epidermis-specific lipoxygenases: 12(R)-lipoxygenase (12R-LOX) and lipoxygenase-3 (eLOX3). These are encoded by ALOX12B and ALOXE3, respectively. When one of these lipoxygenases mutates to an inactivate form, autosomal recessive congenital ichthyosis results [37].

Both 12R-LOX and eLOX3 are dioxygenases that mainly work in the epidermis [38, 39]. Severe skin barrier impairment was reported in 12R-LOX-deficient mice [40]. Lipid analysis elucidated that the oxidized species of linoleic acid in the ceramides are almost absent in the epidermis of 12R-LOX-deficient mice [40]. In addition, epidermal ceramides that were covalently bonded to the CCE significantly decreased to only a few percent in 12R-LOX-knockout mice and to approximately half the normal levels in eLOX3-knockout mice [40-42].

The oxygenation of O-linoleoyl-ω-hydroxy-ULC-glucosylceramide produces an “oxygen signal” in the linoleic acid of ULC-acylglucosylceramide. The “oxygen signal” indicates the linoleic acid as the more polar carbon chain and this signal is required to facilitate the ester hydrolysis.

3.9. Hydrolysis of oxidized linoleic acid

The “oxygen signal” produced by the action of 12R-LOX and eLOX3 in linoleic acid makes the linoleic acid the more polar carbon chain. The oxidized linoleic acid no longer fits in the lipid environment [43]. It is hypothesized that the oxidized linoleic acid serves as a substrate for an esterase/hydrolase that cleaves the oxidized linoleic acid to form ω-hydroxyacyl-sphingosine (OS) with a free ω-hydroxyl [43]. The hydrolase/lipase engaged in this process remains unknown, and no human disease associated with this step has been reported.

3.10. Covalent linking of glucosylceramide to the outer surface of the CCE

The CCE is a 15-nm-thick layer of protein on the inner surface of the cell membrane in granular layer cells. The CCE is essential for barrier function of the stratum corneum [1]. The CCE is formed by the assembly of CCE precursor proteins including involucrin, loricrin and small proline-rich proteins [44]. Epidermal TGases are considered to work at least in part in the crosslinking of CCE precursor proteins [45]. In 1995, Candi *et al*. [46] reported that TGase 1 crosslinks CCE precursor

proteins including involucrin and loricrin to the inner surface of the cell membrane, resulting in CCE formation. Later in the keratinization process, it is hypothesized that TGase 1 also catalyzes the covalent bonding of ω-hydroxy-ceramides to the outer surface of the CCE [10].

Epidermis-specific ceramides are unique, because their ultra-long-chain, C28-C36, acyl chains are precisely long enough to cross the cell membrane lipid bilayer, with the ω-hydroxyl group on the side opposite the sphingosine part. The ω-hydroxyl group is used for covalent bonding to involucrin and other CCE precursor proteins. Crosslinking enzymes, possibly TGase 1, crosslink the ω-hydroxyl onto adjacent early CCE precursor proteins such as involucrin or membrane constituents, including envoplakin and periplakin [10]. The sphingosine hydroxyl is not used for the crosslinking [10].

The fact that LI patients with absent or low TGase 1 activity/protein levels exhibit normal levels of protein-bound ceramides raises questions about the putative role of TGase 1 in crosslinking between ULC-glucosylceramide and CCE [11]. An unknown crosslinking enzyme(s) other than TGase 1 may work in the crosslinking of ω-hydroxyl-ceramides to CCE.

3.11. Deglucosylation of glucosylceramide

By β-glucocerebrosidase, the glucosylceramide is hydrolyzed to ceramide and glucose outside the keratinocyte. The processing of glucosylceramide to ceramide is essential for the formation of both the CLE and the intercellular lipid layers in the stratum corneum. Glucose is removed from ULC-glucosylceramide by the action of β-glucocerebrosidase probably after covalent bonding of ULC-glucosylceramide to CCE, because CCE-bound ULC-glucosylceramides accumulate in β-glucocerebrosidase-deficient mice (type 2 Gaucher disease model mice) and in β-glucocerebrosidase activator protein (saposin-C) knockout mice [47]. The order of crosslinking of ULC-ceramide to CCE and the elimination of glucose are probably

not stringent [8].

Gaucher disease is an autosomal recessive disease caused by a deficiency of glucocerebrosidase β [48]. This deficiency leads to a failure in the hydrolysis of glucosylceramides to ceramides, resulting in the accumulation of excess glucosylceramide in the lysosomes of reticuloendothelial cells and in the involvement of splenic, hepatic, bone and central nervous systems [48]. Patients with Gaucher disease type 2 are reported to show a collodion baby phenotype [49, 50]. The ichthyosis phenotype in these patients is due to severe disease and low residual glucocerebrosidase β enzyme activity in Gaucher disease type 2 [49, 50].

4. Conclusions

In the last two decades, especially in the era of whole-exome/genome sequencing by next-generation sequencers, our knowledge of the causative genes/molecules and pathogenetic mechanisms of ichthyoses has progressed tremendously. The mechanisms of the synthesis, metabolism and dynamics of epidermal lipids, mainly ceramides, and the pathogenesis of ichthyoses have rapidly been clarified in tandem. Investigations on the pathomechanisms of ichthyoses have provided novel knowledge on the synthesis and metabolism of ceramides in the epidermis, and vice versa. The details of ceramide synthesis and metabolism that were clarified recently may lead to innovative therapies for ichthyoses and ichthyosis syndromes. In this context, we assume that research on epidermal ceramides and the pathogenesis of ichthyoses are opening up a new era of skin biology: the lipid era.

We now have a number of potent strategies for innovative novel treatments for genetic disorders, such as genome editing by crisper-Cas and tissue reconstruction using iPS cells. Luckily, we can easily access the skin to test novel therapeutic approaches. Thus, from our knowledge on the pathogenetic mechanisms of

ichthyoses associated with lipid metabolism and transport, I am confident that innovations will be realized in the near future for highly effective treatments of ichthyoses and ichthyosis syndromes through the manipulation of epidermal lipid dynamics.

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Figure legends

Fig. 1. The four major components of the skin barrier in the stratum corneum; 1) keratin, filaggrin and their degradation products, 2) cornified cell envelope (CCE), 3) corneocyte lipid envelope (CLE), 4) intercellular lipid layers. The CLE, a thin, single layer of ULC-ceramide or ULC fatty acid, is essential for sound skin barrier function.

Fig. 2. The pathway of ULC-acylglucosylceramide synthesis in the keratinocyte. Activities of a key enzyme at each step are indicated by dotted arrows. The enzymes and transporter whose deficiencies are known to cause ichthyoses or ichthyosis syndromes are in red. The green arrows indicate linoleic acid supply for ω-O-esterification.

Fig. 3. The formation of the CLE during the late differentiation of keratinocytes: ① the oxidation of linoleic acid in ULC-acylglucosylceramide, ② the hydrolysis of oxidized linoleic acid, ③ the covalent linking of ULC-glucosylceramide to the outer surface of the CCE, ④ the deglucosylation of glucosylceramide*. *For the deglucosylation of ULC-glucosylceramide, the timing might not be necessarily after the covalent bonding of ULC-ceramide to the CCE.