Fat in the skin
Triacylglycerol metabolism in keratinocytes and its role in the development of neutral lipid storage disease

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Introduction

Survival in a terrestrial environment necessitates the prevention of water and electrolyte loss through the body surface to minimize desiccation. In mammals, the epidermal permeability barrier in the cornified layer of the skin (stratum corneum) provides this function. The barrier is built during keratinization and consists of terminally differentiated keratinocytes (corneocytes), which are connected by protein cross-linking. These large aggregates are embedded in a lipid-containing extracellular matrix that mediates permeability barrier function. This prevents the excessive loss of body fluids but in addition also provides an effective barrier against environmental xenobiotics and host infections by pathogens. The most common lipids found in the extracellular matrix of the stratum corneum are ceramides, cholesterol and non-esterified fatty acids (FAs). They originate from enzymatic conversion of glucosylceramides, sphingomyelins, glycerophospholipids and cholesterol sulfate, which are secreted by lamellar bodies from keratinocytes at the stratum granulosum/stratum corneum interphase. Triacylglycerols (TAGs) are a minor lipid species of lamellar bodies and as a result epidermal TAG metabolism did not obtain much attention in the past. Recent discoveries, however, demonstrated that dysfunction and deficiencies of enzymes and activators of TAG synthesis and catabolism cause major skin defects in mice and humans. These findings summarized in this review suggest a crucial role of neutral lipid metabolism in the generation of a functional permeability barrier in the skin.

Neutral Lipid Storage Disease, a Phenotypically Heterogeneous Disorder

The first indication that neutral lipid metabolism may be linked to skin function was obtained in 1966 when Rozenszajn et al. reported on two sisters suffering from neutral lipid storage disease (NLSD) and severe ichthyosis. Later, Dorfman et al. and Chanarin et al. presented additional cases of NLSD patients with ichthyosis and the condition was subsequently termed Dorfman-Chanarin syndrome (DCS). NLSD is defined as a rare
autosomal recessive disorder characterized by excessive deposition of neutral lipids in multiple organs of the body. The very first case report about a patient suffering from NLSD was published in the early 1950s. In this report, Jordans documented the presence of large neutral lipid-containing vacuoles in white blood cells (“Jordans’ anomaly”) of two brothers. Both individuals also suffered from a rapidly progressing skeletal and cardiac myopathy, as well as liver steatosis. In contrast to the patients reported by Dorfman et al. and Chanarin et al., these brothers showed no signs of ichthyosis or other skin defects suggesting genetic and clinical heterogeneity among NLSD patients. To date, a total of more than 50 NLSD cases have been reported. Besides the consistently occurring multi-systemic accumulation of TAGs in these patients, the more inconsistent clinical manifestations of the disorder include ichthyosis, skeletal and cardiac myopathy, liver steatosis with hepatosplenomegaly, neurosensory hearing loss, ataxia, horizontal nystagmus, mental retardation or developmental disorders, such as short stature, microcephalus and ear abnormalities.

Early biochemical studies with cultured skin fibroblasts derived from DCS patients demonstrated that the total cellular TAG content was up to 20-fold higher than in controls, even when cells were cultivated in lipid-deficient media. Biochemical experiments revealed normal FA uptake, transport and β-oxidation. With regard to TAG and phospholipid metabolism, two biochemical defects were identified: Williams et al., Salavayre, Douste-Blazy and colleagues concluded from their studies that TAG accumulation in NLSD fibroblasts is due to a functional deficiency of a TAG lipase. In contrast, Igal and Coleman found that TAG hydrolysis was normal in NLSD fibroblasts but instead demonstrated that acylglycerol recycling to phospholipids was defective and caused increased DAG reesterification and TAG formation. However, the actual gene(s) and protein(s) causing NLSD remained unidentified.

Mutations in the Gene Encoding “Comparative Gene Identification-58” Cause NLSD with Ichthyosis

Important progress was made in 2001, when the group of Fischer and colleagues found several causative mutations in the gene coding for “comparative gene identification-58” (CGI-58) in patients with DCS. Currently, more than two dozen different mutations in the human CGI-58 gene are known including missense mutations, nonsense mutations, splice site mutations at exon-intron boundaries and insertion/deletion mutations. Importantly, all known NLSD patients, who are affected by mutations in the CGI-58 gene, suffer from ichthyosis. The ichthyosiform phenotype of DCS most closely resembles non-bullous congenital ichthyosiform erythroderma and is characterized by a pronounced defect in the permeability barrier function of the skin leading to an increased transepidermal water loss.

As the name implies, CGI-58 was originally identified in a comparative-gene-identification approach, where the human proteome and the proteome of the nematode Caenorhabditis elegans (C. elegans) were aligned to detect orthologous proteins. In the human genome, the CGI-58 gene is located on chromosome 3 and consists of 7 exons spanning a region of about 31.8 kb. Translation of the 1,050 bp mRNA results in a protein composed of 349 amino acids with a molecular mass of approximately 39 kDa. The human protein shares an amino acid homology of 94% with its murine orthologue. The highest expression of CGI-58 is observed in mature adipocytes and testes, but lower levels are also detected in liver, skeletal and cardiac muscle, skin and brain.

Despite the causal relationship between mutations in CGI-58 and the development of DCS, the biochemical function of the protein remained elusive for a long time. CGI-58 belongs to the esterase/thioesterase/lipase subfamily of proteins, structurally defined by the presence of an α/β hydrolase fold. Accordingly, CGI-58 is also annotated as α/β hydrolase domain containing 5 (ABHD5). Unlike all other members of this protein family, however, the putative active serine within the canonical esterase/lipase GXXG sequence motif of the catalytic triad is replaced by an asparagine residue in the corresponding sequence region of CGI-58 (amino acid residue 153 of the human protein). As a result, CGI-58 does not exhibit lipase/esterase activity in vitro studies.

An important function of CGI-58 was discovered in 2006. In this study Zechner and colleagues demonstrated that efficient hydrolysis of TAGs by adipose triglyceride lipase (ATGL) depends on the presence of CGI-58. ATGL is an essential enzyme for the hydrolytic degradation of TAGs, a process commonly named lipolysis. The complete catabolism of TAGs to FAs and glycerol requires the hydrolytic activities of three specific lipases. Adipose triglyceride lipase (ATGL; also termed desnutrin, patatin-like phospholipase domain containing 2, PNPLA2, or transport secretion protein-2, TTS-2) exhibits its high substrate specificity for TAGs and catalyzes the initial step in lipolysis by removing the first FA from TAG molecules thereby generating diacylglycerols (DAGs). Hormone-sensitive lipase (HSL) is the major lipase in DAG hydrolysis, leading to the formation of monoacylglycerols (MAGs) and FAs. In the final step of lipolysis, monoglyceride lipase (MGL) hydrolyzes MAGs to glycerol and FAs. CGI-58 interacts with ATGL, thereby stimulating its enzymatic activity up to 20-fold. Mutant variants of CGI-58, harboring amino acid substitutions, which lead to DCS, completely lose their ability to activate ATGL-mediated lipolysis. Conversely, expression of functional CGI-58 in DCS fibroblasts restored TAG hydrolysis and reversed the lipid storage phenotype in these cells. Thus, CGI-58 appears to be an essential regulatory factor for efficient cellular TAG catabolism. Defective TAG catabolism also provided a plausible biochemical explanation for the multi-systemic TAG accumulation in patients lacking functional CGI-58.

Mutations in the ATGL Gene Cause NLSD with Myopathy but not Ichthyosis

The functional link between CGI-58 and ATGL suggested that in a subpopulation of patients NLSD may be actually caused by mutations in the ATGL gene (PNPLA2). Indeed, in 2007 Fischer et al. published several mutations in PNPLA2 showing
that ATGL dysfunction causes NLSD. To date, 14 patients with 9 different mutations have been reported in references 53–60. All patients exhibit the classical symptoms of multi-systemic TAG accumulation. Surprisingly, however, NLSD patients with mutations in the ATGL gene do not develop ichthyosis. Instead, these patients, compared to those with mutated CGI-58, suffer from more severe forms of skeletal and cardiac myopathy. The cardiac myopathy can be lethal and requires heart transplantation in many cases. In consideration of the phenotypical differences due to distinct genetic defects, Fischer et al.53 suggested the name “NLSD with myopathy” (NLSDM) for the subgroup of patients lacking normal ATGL function and “NLSD with ichthyosis” (NLSDI) for those patients, who are affected by CGI-58 mutations (previously termed DCS).

The clinical symptoms of NLSDM resemble in many ways the phenotype of ATGL-deficient mice, i.e., excessive accumulation of TAG in multiple tissues and cell types, including heart, skeletal muscle, liver and lymphocytes. Notably, ATGL-deficient mice just like humans suffering from NLSDM also do not display ichthyosis or other forms of skin defects. This argues against the involvement of ATGL in the development of ichthyosis and provides strong evidence for an ATGL-independent function of CGI-58 in epidermal lipid metabolism.

### The ATGL-Independent Function of CGI-58 in the Skin: Role of Epidermal TAG Hydrolysis

An ATGL-independent function of CGI-58 was evident, when Radner et al.62 reported on the phenotype of mice lacking CGI-58. Similar as observed in patients suffering from NLSDI, newborn CGI-58-deficient mice suffer from an ichthyosiform skin defect. In contrast to NLSDI patients, however, the defect is much more severe leading to rapid desiccation and premature death within a few hours after birth. The reason for this phenotypical difference between CGI-58-defective humans and mice is currently unknown. A plausible explanation could be that humans have a more advantageous ratio of body volume to skin surface, impeding rapid dehydration. Indirect support for this speculation came from a recently published NLSDI case report on a patient with strong seasonal variation of disease activity.39 Ichthyosiform skin lesions in this subject recurred every year with beginning of summer and mostly disappeared with lower air temperatures in autumn and winter indicating more pronounced severity of disease symptoms with higher ambient temperatures.

CGI-58 knockout mice also provided a suitable model to address questions regarding the biochemical defects leading to barrier dysfunction. This study demonstrated reduced TAG hydrolase activity in association with increased TAG accumulation and the absence of measurable concentrations of ω-(O)-acylceramide (acycleramides) species in the CGI-58-deficient epidermis.62 The defect could be partially restored by the addition of purified CGI-58, but not by ATGL. These observations suggest that CGI-58 activates a TAG-lipase distinct from ATGL, which is required for the provision of FAs for the esterification of ω-OH-ceramides to generate acycleramides. Acycleramides are critical for the covalent binding of ω-OH-ceramides to structural proteins of corneocytes (involucrin, envoplakin and periplakin).63–65 This layer of protein-bound ω-OH-ceramides is referred to as corneocyte lipid envelope (CLE) and provides an interface between the cross-linked corneocyte proteins and the lipid-containing extracellular matrix. The generation of acycleramides and their involvement in the development of a functional CLE is summarized in Figure 1. Acycleramides are formed from ceramides by amide bonding of very-long chain FAs to sphinganine,
subsequent ω-hydroxylation of these FAs, and esterification of the ω-OH group, mostly by linoleic acid. Wertz and Downing have demonstrated in porcine epidermis that the majority of linoleic acid for acylceramide synthesis originates from phospholipids and TAGs. In CGI-58-deficient mice this transfer apparently does not occur, leading to the accumulation of ω-OH-ceramides and the absence of acylceramides and its secondary products, like protein-bound ω-OH-ceramides (Fig. 1).

Importantly, these results conform to observations in the epidermis of NLSDI patients. In humans CGI-58 co-localizes in the stratum granulosum with lamellar bodies. Lamellar body contents are secreted from keratinocytes into stratum corneum interstices and further metabolized by co-secreted enzymes, including TAG hydrolase(s), β-glucocerebrosidase, sphingomyelinase, and phospholipase(s). These enzymes are essential for the generation of the lipid constituents of the epidermal barrier. CGI-58 deficiency in human epidermis results in abnormal lamellar bodies in the granular layer containing neutral lipid micro-inclusions that are co-secreted with lamellar contents. As a consequence, the excessive incorporation of non-polar lipids into lamellar membranes forms a non-lamellar phase, which markedly impairs permeability barrier function (Fig. 1). Taken together, human CGI-58-deficient epidermis lacks an essential TAG hydrolase activity to catabolize lamellar TAGs. Just as in CGI-58-deficient mice, the absence of this lipase activity causes a lack of acylceramides and protein-bound ω-OH-ceramides in the epidermis of NLSDI patients arguing for a defective FA transfer from TAG (or phospholipids) to ω-OH-ceramides.

Using the ORESTES (open reading frame ESTs) method, an excellent study investigating human genes involved in epidermal differentiation and barrier function, revealed a list of several potential candidates for the postulated unknown epidermal TAG lipase. The authors identified in the human genome many potential lipase genes including members of the lipase-like family (LIPIK, LIPM and LIPN), the PNPLA family (PNPLA1), the family of esterases/carboxyl esterases (CES3) and the ABHD family (ABHD9). Whether any of these genes encode lipases involved in TAG hydrolysis in a CGI-58-dependent manner remains to be explored. The classical enzymes of lipolysis (ATGL, HSL and MGL) were not detected in this study. In addition, no information on expression levels of these enzymes in the epidermis was reported. Thus, these enzymes are not expected to play a major role in epidermal TAG catabolism.

The immanent role of epidermal TAG stores in acylceramide synthesis is also evident from a mutant mouse model lacking the principal enzyme for epidermal TAG synthesis, acyl-CoA:diacylglycerol acyltransferase-2 (DGAT-2). DGAT-2-deficient mice also exhibit drastically reduced epidermal acylceramide levels and increased transepidermal water loss due to a severe permeability barrier dysfunction of the skin. This phenotype is essentially identical to the one observed in CGI-58-deficient mice and also leads to postnatal death within a few hours after birth. In contrast to CGI-58-deficient mice, however, the skin defect in DGAT-2-deficient mice seems to include a systemic component, because transplantation of full-thickness DGAT-2-deficient skin onto the back of athymic nude mice showed a correction of the epidermal structural and functional defects. A similar transplantation experiment with CGI-58-deficient mice failed to correct the skin phenotype arguing for a skin autonomous pathology. Further studies are required to elucidate this interesting difference between the two mouse models. Nevertheless, the phenotypes of CGI-58- and DGAT-2-deficient mice suggest that in the epidermis FAs have to cycle through esterification (TAG synthesis) and re-hydrolysis (TAG degradation by lipases) in order to be available for ω-OH-ceramide esterification (Fig. 1).

The ATGL-Independent Function of CGI-58 in the Skin: CGI-58 is an Acyl-CoA:Lysocephatidic Acid Acyltransferase

In 2008, Gosh et al. reported that CGI-58 exhibits enzyme activity as an acyl-CoA-dependent lysophosphatidic acid acyltransferase (LPAACT) generating phosphatic acid. This activity was later confirmed by several other groups. Phosphatic acid is a key metabolite in the synthesis pathway of both TAGs and glycerophospholipids and it is therefore conceivable that the CGI-58-associated LPAT activity relates to the skin phenotype in NLSDI patients and mice with CGI-58 deficiency. Additionally, this activity coheres with previous studies by Igol and Coleman identifying a defect in the remodeling of TAGs to glycerophospholipids in skin fibroblasts of patients affected by NLSDI (see above). To assess whether CGI-58 affects epidermal phospholipid metabolism, Radner et al. analyzed LPAT activity and phospholipid composition of normal and CGI-58-deficient epidermal explants. In keratinocytes, phospholipids are usually secreted by lamellar bodies and subsequently hydrolyzed by secretory phospholipase A2 activity in the cornified layer. Consequently, a lack of phospholipids in the epidermis would deplete the extracellular, non-esterified FA pool and impair stratum corneum acidification. However, CGI-58-deficient mice exhibited normal LPAACT activity in skin lysates, normal epidermal phospholipid composition, and normal acidification of the stratum corneum (unpublished observation). These findings argue against a major role of CGI-58 in epidermal phospholipid metabolism.

Additionally, it is unlikely that the LPAACT activity of CGI-58 contributes to the net synthesis of TAG in keratinocytes because CGI-58-deficiency in humans and mice causes TAG accumulation. This is in contrast to the clinical appearance of patients lacking classical LPAACT enzymes such as acyl-CoA:acylglycerol acyltransferase (AGPAT). All of these patients suffer from the severe lack of TAGs and lipodystrophy. Moreover, mutant proteins of CGI-58 as present in patients with NLSDI retain full LPAACT activity in vitro. However, it is conceivable that the acyltransferase activity of CGI-58 is confined to specific cellular compartments (e.g., lipid droplets or lamellar bodies) and/or substrates. For example, it may be relevant for the production of signaling lipids (e.g. phosphatidic acid) that affect keratinocyte differentiation or acylation of alternative lipid substrates, such as ω-OH-(glucosyl) ceramides, to generate acyl(glycosyl) ceramides. Although such
an enzyme activity has been described in the epidermis the corresponding gene and protein remain unidentified. The Role of CGI-58 in Keratinocyte Differentiation

Epidermal differentiation is a highly regulated, sequential process, which starts with the migration of keratinocytes from the proliferating, basal cell layer of the epidermis through the spinous and granular layer and ends with the formation of the stratum corneum.\textsuperscript{90,91} Using both quantitative real-time reverse transcription polymerase chain reaction and immunohistochemistry, Akiyama et al.\textsuperscript{67} demonstrated a crucial role of CGI-58 in keratinocyte differentiation by showing that epidermal CGI-58 transcription is massively upregulated during cornification and fetal development of human skin. In accordance with these observations, a reduced expression of keratinization markers was observed in CGI-58 silenced keratinocytes (via siRNA) and murine CGI-58-deficient epidermis.\textsuperscript{62,67} The mechanisms by which CGI-58 affects these biological processes remain speculative but it is conceivable that CGI-58 is involved in the generation of lipid ligands for nuclear hormone receptors that regulate keratinocyte differentiation. Human epidermis expresses all isoforms of peroxisome proliferator-activated receptor (PPAR, isoforms: PPAR-alpha, PPAR-beta/delta, PPAR-gamma), retinoid X receptor (RXR, isoforms: RXR-alpha, RXR-beta, RXR-gamma), as well as liver X receptor (LXR, isoforms: LXR-alpha, LXR-beta).\textsuperscript{92-94} Activation of these receptors induces epidermal differentiation and improves permeability barrier homeostasis by stimulating epidendial lipid synthesis, lamellar body formation and secretion of lipids and enzymes, which are required for processing of extracellular lipids in the cornified layer that facilitate adequate lamellae membrane formation and consequently, normal permeability barrier function (Fig. 1).\textsuperscript{95}

According to the current concept, PPARs and LXRs form heterodimers with RXRs. Ligand-binding causes the dissociation of co-repressors and the concurrent recruitment of co-activator proteins to the complex. This, in turn, promotes transcription of downstream target genes.\textsuperscript{96,97} Endogenous lipid-ligands for PPARs, LXRs, as well as RXRs include FAs, oxysterols and 9-cis retinoic acid, respectively. CGI-58 may contribute to the generation of these lipids. For example, the activation of TAG hydrolysis by CGI-58 may provide FAs for PPAR activation. The LPAAT activity of CGI-58 may lower the cellular concentration of lysophosphatidic acid and increase the levels of phosphatidic acid, thereby also affecting PPAR activity. 9-cis retinoic acid, the lipid ligand for RXR activation, derives from retinyl ester stores in keratinocytes. Its generation requires retinyl ester hydrolysis and retinol oxidation in a two-step process. Notably, retinyl ester hydrolysis in human epidermis is mediated by PNPLA4 (also annotated as gene sequence 2, GS2), a protein with high structural homology to ATGL.\textsuperscript{98,99}

Conclusion

Recent advances in the understanding of epidermal differentiation and the formation of the skin permeability barrier acknowledge the immanant role of TAG-derived FAs in these processes. Studies with mutant mouse models and patients with NLSDI demonstrated that FAs originating from the lipolytic hydrolysis are a prerequisite for the formation of acyl(glucosyl)ceramides, which are essential for the formation of a functional CLE. However, important questions concerning the role of neutral lipid metabolism and epidermal lipid remodeling remain unanswered. For example, it is unclear why FAs must derive from TAGs for acylceramide synthesis and which CGI-58-regulated lipase mediates the hydrolysis of TAGs in keratinocytes. Future studies are required to elucidate how various activities of CGI-58 affect the formation of bioactive lipids that are involved in cellular signaling. These processes may include FA or phosphatidic acid activation of nuclear hormone receptors, which are known to control the complex and essential differentiation of keratinocytes during permeability barrier development.

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