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Role of Ceramide in the Barrier Function of the Stratum Corneum: Implications for the Pathogenesis of Atopic Dermatitis

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Abstract

Atopic dermatitis is a recurrent dermatitis which is characterized clinically by atopic dry skin and functionally by cutaneous barrier disruption even in the non-lesional skin. These abnormalities have been thought to be mainly attributable to significantly decreased levels of ceramides even in the non-lesional stratum corneum. Recently, in association with the barrier disruption in atopic dermatitis skin, prevalent and rare loss-of-function mutations in the gene encoding filaggrin have been reported to be an important pre-disposing factor for the development of atopic dermatitis. However, a mechanistic connection between filaggrin loss-of-function mutations and barrier disruption has not been resolved and remains controversial. This review article explores the physiological and biochemical basis for the atopic dermatitis phenotype in terms of the barrier abnormality and associated factors such as ceramides and its metabolites in the following sequence: 1. Clinical characteristics as a recurrent dermatitis, 2. Abnormality in cutaneous permeability barrier function, 3. Is barrier disruption a cause or a result of dermatitis?, 4. Is the barrier abnormality inherent or not? 5. Role of ceramides, 6. Transglutaminase or filaggrin mutations and barrier disruption, 7. Contribution of the barrier abnormality to the Th1/Th2 balance, 8. Th1/Th2 balance and ceramide deficiency, 9. *S. aureus* colonization and ceramide deficiency, 10. Is the ceramide deficiency inherent or not ?, 11. Inflammation as a causative factor that down-regulates ceramide generation, 12. Biological mechanisms underlying the ceramide deficiency, 13. Clinical efficacy of ceramide as a major role for the pathogenesis of atopic dermatitis.

Abbreviations: TEWL: Transepidermal Water Loss; AD: Atopic Dermatitis; SC: Stratum Corneum; HC: Healthy Control; PiCl: Picryl Chloride; MA: House Dust Mite Antigens; SM: Sphingomyelin; GCer: Glucosylceramide; BGCase: Beta-Glucocerebrosidase; SMase: Acid Sphingomyelinase; CBE: Conduritol B Epoxide; DSP: Despiramine Hydrochloride; PDMP: 1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol; SPT: Serine Palmitoyl Transferase; CDase: Acid Ceramidase; SPC: Sphingosylphosphorylcholine; GSP: Glucosylsphingosine; HIRU: Mucopolysaccharide; CER: Synthetic Pseudo-Ceramide

Clinical Characteristics as a Recurrent Dermatits

The skin of patients with atopic dermatitis (AD) is mainly characterized by a clinically normal appearance but which exhibits a high susceptibility to irritants and allergens, dry skin and deficient innate immunity. Thus, as shown in Figure 1, an increased transepidermal water loss (TEWL) and decreased water content, even in the clinically normal skin of patients with AD suggest a marked down-regulation of both the barrier and the water holding functions. AD skin also shows a high frequency of *S. aureus* colonization even in the nonlesional skin, compared with no colonization in healthy control (HC) skin [1-4].

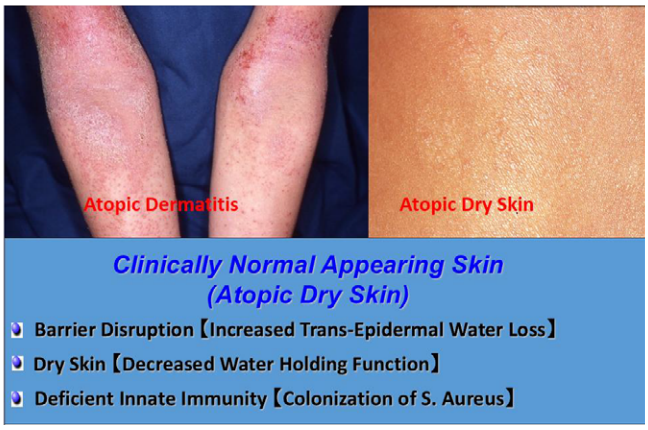

Figure 1: Clinical Characteristics of Atopic Dermatitis which are Distinct from Other Types of Dermatitis [1,2].

Abnormality in Cutaneous Permeability Barrier Function

There is a general consensus that disruption of the barrier function of the stratum corneum (SC) is an essential etiologic factor for skin inflammation in patients with AD. Thus, AD could be considered as a barrier disease in which antigens and irritants that permeate the skin trigger and worsen the dermatitis. Since TEWL, which is frequently evaluated as an indication of barrier function, is not necessarily a precise reflection of cutaneous permeability, we determined whether chemical penetration is really enhanced or not in the nonlesional skin of AD patients compared with HC skin [5]. To evaluate in vivo cutaneous permeability, we used photoacoustic spectrometry by which chemical concentrations present through the layers of the SC can be measured using the intensity of photoacoustic signals derived from the chopped expansion of air due to chemical heat released from chemical molecules

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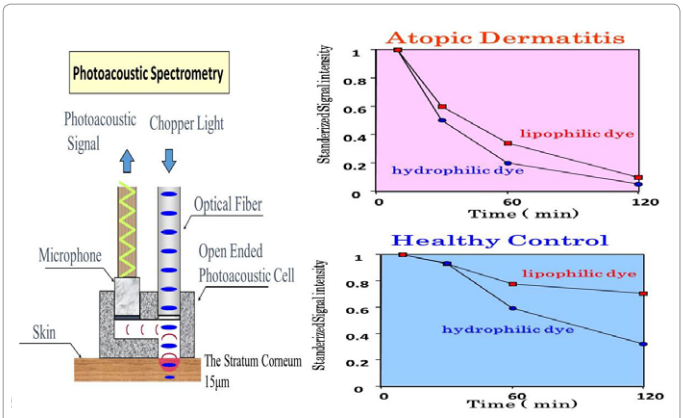

Figure 2: Penetration Profile of Lipophilic and Hydrophilic Dyes through the Stratum Corneum of Forearm Skin as Measured by Photoacoustic Spectrometry [5].

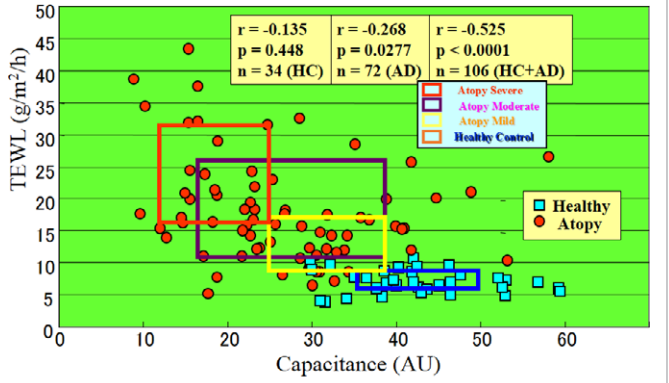

Figure 3: Relation between TEWL and Capacitance Values in Association with the Severity of Atopic Dermatitis [6].

excited by the chopper light. As penetrators, rodamine B stearate (Red 215) and tartrazine (Yellow 4) are used as liphophilic and hydrophilic dyes, respectively, and there in vivo penetration rates through the SC are measured as photoacoustic signals which are equivalent to chemical concentrations within 15 μm thick SC layers. As shown in Figure 2, we found that both dyes penetrate faster in the clinically normal skin of patients with AD compared with HC skin, an indication that there is impairment in the in vivo cutaneous permeability barrier function against lipophilic and hydrophilic chemicals. To reduce the long time required (up to 2 hours) to measure the disappearance rate of chemicals through the SC layers, we used a patch chamber applied on the skin for 2 min and measured the concentrations of chemicals that penetrated the SC layers. Photoacoustic spectrometric analysis after application of the two dyes for 2 min under the closed patch conditions revealed that there was a significant increase in the photoacoustic signals equivalent to dye concentrations through the SC for both lipophilic and hydrophilic dyes in the clinically normal skin of AD patients (n=103) compared with HC skin (n=10). This indicates that the clinically normal skin of patients with AD has accelerated penetration rates for both types of dyes compared with HC skin. It is well known that serum IgE levels represent a hallmark of the atopic diathesis, and about 80% of AD patients have increased levels of serum IgE. Our results indicated that AD patients who have a higher penetration against hydrophilic substances in the nonlesional AD skin tend to exhibit higher IgE levels, resulting in severe dermatitis. Since soluble protein antigens, such as mite antigens (MAs) are major allergens in AD, this suggested that the barrier disruption is a major causative factor for allergic dermatitis due to MAs which may dissolve in sweat.

Is Barrier Disruption a Cause or a Result of Dermatitis?

To determine whether disrupted barrier function in the nonlesional skin of AD patients is associated with pre-inflammatory and/or post-inflammatory events, which are relevant to the severity of AD or local dry skin properties, respectively, we evaluated the barrier function and the water content of nonlesional forearm skin and compared these with the severity of AD [6]. TEWL in the nonlesional AD skin was significantly increased in proportion to the severity of AD with a markedly high correlation coefficient ($r=0.834$, $p<0.0001$, $n=106$), when the HC, and the mild, moderate and severe groups of AD are assigned as 0, 1, 2 and 3, respectively. This indicates that the barrier disruption in nonlesional AD skin is well suited to reflect the severity of AD and that the nonlesional AD skin had already been inflamed and has never been the same as HC skin since birth. When the relationship between the disease severity of AD and the water content measured in the nonlesional AD skin was assessed, there was also a significant correlation ($r=-0.720$, $p<0.0001$, $n=106$) between the capacitance values and the disease severity of AD in which the difference between the conductance values of the HC skin and the mild AD skin is significant ($p<0.01$). As shown in Figure 3, comparison between TEWL and capacitance in association with the disease severity of AD reveals that those 2 characteristics are correlated with the severe, moderate and mild groups of AD skin and HC skin. Therefore, our results indicate that the barrier disruptions as well as the water deficiency in the nonlesional AD skin reflect the disease severity of AD. Further, the combination of barrier and water-holding functions in the nonlesional AD skin correlated with the disease severity of AD and suggests that the barrier abnormality as well as the water deficiency are elicited as a result of the dermatitis and also subsequently trigger the recurrence of dermatitis.

Is the Barrier Abnormality Inherent or not?

It is important to ask whether impairment of the skin barrier function in AD is inherent or not. In this respect, a prospective study using newborns revealed that impairment of the skin barrier function was not inherent in AD patients [7]. Consistent with non-inherent and post-inflammatory events, analysis of SC function in the skin of infants with AD (n=22~28), where the nonlesional AD skin can be considered similar to the intact skin compared with adult AD skin revealed that while TEWL is significantly up-regulated in the AD lesional skin, but not the nonlesional skin compared with HC skin (n=18~20), the water content is slightly but not significantly decreased only in the lesional skin [8]. In the buttock skin, both TEWL and water content did not differ between the AD nonlesional skin and the HC skin (n=18~20). The sum of these findings suggests that the barrier abnormality is not inherent.

Role of Ceramide

Our previous studies have shown that the barrier disrupted dry skin of AD patients is mainly attributable to significantly decreased levels of ceramides (evaluated as μg ceramide/weight or protein) in the SC [9]. Ceramide acts as a water modulator and a permeability barrier by forming multi-layered lamellar structures with other lipids between cells in the SC layers [10,11]. In adult AD skin, there is a ceramide deficiency even in the nonlesional SC, which is highly associated with the abnormal barrier function, predisposing the skin to inflammatory

processes evoked by irritants and allergens. The ceramide deficiency in the SC of AD skin has been substantiated in many additional studies [3,12-16]. The significance of the deficit in ceramides in the SC that impairs the cutaneous permeability barrier was evidenced by the clinical observations that TEWL assessed in AD nonlesional skin increases inversely with the decreased levels of ceramides in the SC from the same skin site of AD patients [1,2]. Serine palmitoyltransferase long-chain base subunit 2-targeted mice showed decreased ceramide levels in the epidermis, which impaired water-holding capacity and barrier function [17]. Dietary sphingolipids was found to improve skin barrier functions via the upregulation of ceramide synthases in the epidermis [18]. Further, the impaired barrier function which occurs in essential fatty acid deficient mice or is elicited by surfactant or solvent treatment can be repaired by the topical application of pseudoceramides [19-21]. The sum of these findings suggests that decreased ceramide levels in the SC are responsible for the defect in barrier function, under which conditions many foreign substances, including MAs, can easily penetrate through the SC to elicit allergic reactions. In addition to ceramide deficiency, changes in ceramide profiles including ceramide chain length was found to be associated with the impaired SC barrier function in AD [16,22,23].

Transglutaminase or Filaggrin Mutations and Barrier Disruption

It has been reported that transglutaminase-1 (TGase 1) (-/-) knockout mice have perturbed skin barrier function [24]. TGase 1contributes to the development of the cornified cell envelope (CCE) [25,26]. CCE serves as a physical and water impermeable barrier and is missing in patients with lamellar ichthyosis (LI) [27]. Keratinocytes of autosomal recessive congenital ichthyosis (ARCI) patients have decreased TGase-1 enzyme activity levels [28,29]. Human epidermal scales of ARCI patients with TGase 1 mutations have a structurally perturbed CCE which exerts defects in skin-barrier function [30]. It seems likely that TGase-1 is an essential bio-factor for normal epidermal barrier function [31,32]. We reported that UVB-induced perturbation of the skin barrier results from decreased levels of covalently bound ceramide, which is accompanied by decreased expression of TGase 1 gene in UVB-exposed epidermis [33]. O-hydroxyceramides are implicated to be ester-linked by the action of TGase 1 to glutamine and to glutamate residues of a number of cell envelope structural proteins, most notably involucrin, which serve as cornified cell envelope proteins bound to ceramide and thus contribute to the barrier function of the epidermis [34,35]. However, there have been no reports to link the barrier disruption in AD to the abnormality of TGase 1.

Recently, in association with the water deficiency as well as the barrier disruption in AD skin, prevalent and rare loss-of-function mutations in the gene encoding filaggrin (FLG) have been identified as the cause of the genodermatosis ichthyosis vulgaris and were additionally reported to be an important pre-disposing factor for the development of AD [36-39]. The mutation pattern and frequency in the FLG gene of patients with AD or ichthyosis vulgaris was found to vary in different countries with a 27% frequency in Japanese AD patients compared with 3.7% of Japanese HCs [40-45]. Consideration of FLG mutations as an important pre-disposing factor for AD is mainly based on the hypothesis that loss-of-function variants of FLG result in the down-regulated levels of its degradation products (amino acids) which are believed to serve in the barrier function due to increased skin hydration. Although there are several reports that support the qualitative relationship between loss-of-function mutations in the FLG gene or filaggrin deficiency and the barrier disruption in AD, this hypothesis lacks reasonable biochemical mechanisms by which FLG mutations elicit the barrier disruption [46-50]. In fact, some reports have contradictorily demonstrated that AD patients exhibit a reduced skin barrier function regardless of the FLG genotype, which suggests that other factors besides loss-of-function mutations in FLG modulate skin barrier integrity [51]. Further, it has been established that the barrier function, as evaluated by TEWL, occurs as an independent factor from skin hydration as evidenced by the facts that subjects with senile xerosis have severe dry skin in spite of the lack of barrier disruption and that treatment of human skin with acetone/ether predominantly elicits dry skin without any increase in TEWL [2,52]. Thus, a mechanistic connection between FLG loss-of-function mutations and barrier disruption has not been resolved and remains controversial. In a search to directly associate FLG mutations with barrier disruption, which is the typical atopic skin phenotype, it has recently been reported that FLG-null mice have no significant abnormality in barrier function or water content as evaluated by TEWL and conductance values, respectively, despite the fact that levels of filaggrin-derived amino acids are markedly reduced in the SC although outside-in cutaneous permeability is disrupted [53]. This suggests that filaggrin-derived amino acids have no distinct contribution to barrier function evaluated by TEWL as well as skin hydration measured by conductance value, which renders the FLG mutation mechanism as a predisposing factor of AD a very complex issue. Another hypothesis that FLG mutations are also linked via an as yet unknown mechanism to decreased ceramide levels in the SC has been challenged and there is no clear relationship between ceramide levels in the SC and FLG mutations in AD patients although FLG deficiency induced in 3 D reconstructed skin has been reported to elicit impaired lipid profile such as an accumulation of free fatty acids (2-fold increase) which leads to less ordered intercellular lipid lamellae and higher permeability [12,22,54]. If mutations in the human FLG gene may provoke a barrier abnormality, yet additional acquired stressors might be necessary because the same mutations in FLG can result in a noninflammatory disorder, ichthyosis vulgaris. Further, DNA mutations, such as FLG mutations, should be highly involved in inherent skin phenotypes as a barrier abnormality, but this is not the case.

Contribution of the Barrier Abnormality to the Th1/Th2 Balance

Since AD patients have constitutive barrier disruption even in their nonlesional skin and since they acquire Th2 dominance in relation to their high sensitivity to various allergens, we assessed the immunological responses in afferent and efferent phases of the sensitization with picryl chloride (PiCl) and house dust MAs through barrier-disrupted skin [55]. Sensitization with PiCl after barrier-disruption of the skin down-regulated the expression of mRNAs for Th1 type cytokines such as IFN-γ and IL-2 without changing the expression of the mRNA for IL-4. The down-regulated expression of the IFN-γ mRNA occurred in proportion to increased TEWL and in a tape-stripping number-dependent fashion without changing the expression of the G3PDH gene. In the serum of DNCB-sensitized mice, there was a significantly increased level of IgE but not of IgG1 or IgG2 a after the barrier disruption by tape-stripping. When mice were sensitized with MAs by a single topical application to barrier-disrupted abdominal skin, there was a marked up-regulation of mRNA expression for the Th2 cytokine, IL-4, but not for the Th1 cytokines, IL-2 or IFN-γ in the lymphnodes. The up-regulated expression of IL-4 mRNA occurs in proportion to an increasing TEWL and in a tape-stripping number-dependent manner without changing the expression of the β-actin gene. Since inflammation with Th2-type immunity is generally characterized by

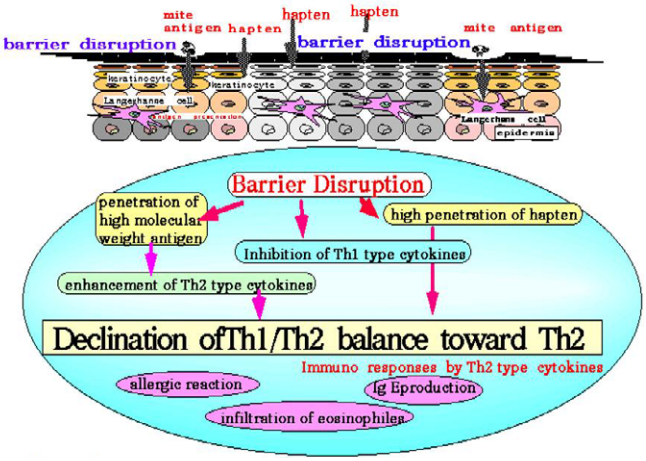

Figure 4: Contribution of the Barrier Abnormality to the Th1/Th2 Balance [56].

the infiltration of eosinophils in the dermis during the elicitation phase, we determined whether allergic reactions evoked by MAs through the barrier-disrupted skin are accompanied by the infiltration of eosinophils in the elicited dermis compared with that evoked by PiCl through intact skin. The results showed that the cutaneous inflammation evoked by MAs in mice sensitized through barrier-disrupted skin was associated with significant infiltration of eosinophils in the dermis as assessed by Biebrich-Scarlet staining. Quantitative analysis revealed that the infiltration of eosinophils elicited by MAs was greater than that elicited by PiCl in mice sensitized through intact skin. Elicitation with MAs or PiCl did not change the number of mast cells or basophils in the elicited aural skin. In summary, as depicted in Figure 4, our results strongly suggest that percutaneous sensitization by a single topical application of haptens or the AD-specific macro molecular antigen, MA, following cutaneous barrier disruption, elicits a Th2-dominant immune response including IgE over-production and eosinophil infiltration. Our immunological study strongly suggests that the Th2 dominance in AD is greatly associated with the constitutive barrier impairment but not with an innate immunological abnormality.

Th1/Th2 Balance and Ceramide Deficiency

As for the mechanism(s) involved in the continuous ceramide deficiency in the SC of AD skin, Th2 type cytokines have been reported to affect the expression of ceramide-producing enzymes, resulting in the reduced levels of ceramide in the epidermis [56,57]. However, almost all related studies have focused on ceramide levels in the whole epidermis but not in the SC layers alone, even though the ceramide level in the SC is predominantly responsible for cutaneous barrier function. Using human epidermal equivalents, we evaluated the effects of Th1/Th2 cytokines on levels of ceramide in the SC. In other studies which used 3-dimensional epidermal equivalents as well as epidermal sheets in culture, it was difficult to assess the effects of chemicals or cytokines on ceramide levels in the SC since the entire SC is already formed before the start of the culture and normal desquamation of the SC layers never occurs in a pattern similar to normal epidermis *in vivo* due to the wet conditions. Because of these constraints, all evaluations conducted on ceramide changes had been limited not to the SC, but to the whole epidermis. Therefore, in our modified method, epidermal sheets without SC layers under a pre-keratinizing condition were cultured for 1 week to generate complete SC layers after which ceramides were extracted from separated whole SC layers and then were quantified as per SC protein. Comparison of the lipid composition by thin layer chromatography shows that the pattern of lipids obtained from the whole SC layers of 3-dimensional epidermal models are almost identical to that from the SC layers of normal human forearm skin. Prior to evaluating the effect of cytokines on ceramide levels in the SC, we evaluated the feasibility of our 3-dimensional epidermal model to examine the effect of an inhibitor for β-glucocerebrosidase (BGCase), bromoconduritol B epoxide (CBE), on ceramide levels and its composition in the SC [58]. The addition of CBE at a concentration of 3 mM during the 7 days of culture resulted in a marked decrease in total ceramide expressed per mg protein. In contrast, there was a remarkable up-regulation of the glucosylceramide (GCer) level. The composition of ceramide species demonstrates that all ceramide species were significantly reduced by the CBE treatment. Taken together with our other similar studies using inhibitors for GCer synthase,1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), andacid sphingomyelinase (SMase), despiramine hydrochloride (DSP), we confirmed that several specific sphingolipid metabolic inhibitors induce a ceramide deficiency in the SC in a fashion similar to the *in vivo* situation. These results underscore the superiority of the reconstructed human epidermal keratinizing equivalent model to examine factors that regulate ceramide synthesis, especially in the human SC, as well as for analyzing their modes of action. We then examined the effects of the Th1/Th2 cytokine balance on ceramide levels in the SC by adding several cytokines to the human keratinizing epidermal equivalents [59]. The addition of a Th1 cytokine (GM-CSF) at a concentration of 10 nM during the 7 days of culture induced no degenerative changes in the epidermis and resulted in a significant increase of the ceramide level expressed as μg/mg protein in the SC. RT-PCR and Western blotting analyses for several sphingolipid enzymes indicated that GM-CSF slightly increased the ceramide level in the SC by up-regulating SMase. In contrast to the Th1 cytokine, the addition of the Th2 cytokine, IL-4, induced exfoliative changes in the epidermis and resulted in a marked decrease of ceramide expressed as μg/mg protein. Analysis of mRNA levels of several sphingolipid metabolic enzymes demonstrated that whereas there was no change in the expression of serine palmitoyl transferase (SPT) I or acid ceramidase (CDase) (at day 4 of culture), mRNAs encoding SPT–II, BGCase and CDase were significantly down-regulated by IL-4. Collectively, our results indicate that IL-4 reduces ceramide levels in the SC by down-regulating SMase and BGCase as well as SPT. In summary, as summarized in Table 1, together with the results for interferon-γ and IL-6, our studies indicate that whereas the addition of Th1 cytokines, such as GM-CSF and IFN-γ results in a distinct increase in SC ceramides by up-regulating ceramide producing enzymes (especially SMase and/or BGCase), Th2 cytokines, such as IL-4 and IL-6, are associated with a marked decrease in SC ceramide by down-regulating SPT-II and BGCase. In conclusion, the sum of the above findings suggests that the Th2 type of inflammation evoked in AD skin is one of the essential factors in down-regulating the levels of ceramides in the SC.

S. aureus colonization and Ceramide Deficiency

Intriguingly, there is a positive relationship between *S. aureus* colonization and the disease severity of AD in both the lesional and the nonlesional skin [4], which suggests that the disease severity predominantly triggers the defense mechanism against *S. aureus* colonization. One sphingolipid metabolite, sphingosine, but not ceramide, is well known to have potent anti-microbial effects on *S. aureus* at physiological levels [60,61]. Thus, it is conceivable that

	stratum corneum			epidermis			gene					protein		activity	
	ceramide/protein	ceramide/well	protein/well	ceramide/protein	ceramide/well	protein/well	SPT1	SPT2	aSMase	BGCase	aCDase	aSMase	aCDase	aSMase	BGCase
GM-CSF	↑	↑↑	→	→	→	→	↗	↗	→	→	→	↑↑	→	↑↑	↑
IFN-γ	↗	↓↓	↓↓				↑↑	↑↑	↑↑	↑↑	↑↑	↘	↘	↓↓	↑↑
TNF-α	↗	↓↓	↓				↑↑	↑	↗	↑↑	↑↑				
IL-4	↓	↓↓	→	↘	↘	→	→	↓↓	↓↓	↓↓	→	↑	↘	↑↑	↓↓
IL-6	↓	↓↓	↓	↓	↑	↑↑	→	↓↓	↓↓	↓↓	↓↓	↓	↓↓	↘	↓↓
IL-8	→	→	→												

Table 1: Effects of cytokines on total ceramide levels, the mRNA and protein expression levels of ceramide metabolic enzymes and their enzymatic activities [60].

sphingosine may play a role, though not exclusively, in the bacterial defense mechanisms of HC skin. It was therefore of particular interest to characterize the level of sphingosine in the SC of patients with AD. Quantitation of sphingosine by radiolabeling reveals that unexpectedly, there is a significant decrease in sphingosine level per mg SC in the lesional and nonlesional SC of patients with AD (n=73~83) compared with HCs (n=69) [3]. There is a close relationship between sphingosine levels and S aureus colonization in which the decreased level of sphingosine is relevant to the increased numbers of *S. aureus* present in the upper SC from the same AD subjects. Comparison between ceramide and sphingosine levels in the same SC also reveals that there is a close correlation between those two parameters in the upper layers of the SC, which suggests that the sphingosine deficiency partly results from the ceramide deficiency. Further, comparison of CDase activity and the sphingosine level in the same SC also revealed that there is a distinct correlation between those two parameters, which suggests that the sphingosine deficiency in AD skin may also result from the decreased activity of CDase. Based on the above results, the colonization of *S. aureus* in AD skin may result from a decrease in the anti-microbial lipid, sphingosine, due to the ceramide deficiency, which is deeply associated with the barrier disruption.

On the other hand, anti-microbial peptides such as defensin have recently been implicated to play an important role in host defense and cutaneous innate immunity [62]. β-defensin-2 was reported to be down-regulated at the gene and protein levels in the whole skin of AD patients compared with psoriasis patients but not with HCs [63]. However, little was known about their role in the colonization of *S. aureus* in the SC from AD patients because a precise evaluation of these peptides as a front-line anti-microbial barrier against *S. aureus* colonization has not been yet performed, especially in comparison with HC skin where no colonization of *S. aureus* exists. Quantitative analysis using the 3 SC strips by western blotting following immunoprecipitation using an anti-β-defensin-2 antibody revealed that whereas the SC from HC skin contains a negligible content of β-defensin-2, the SC from AD, psoriasis and contact dermatitis skin has markedly increased levels [4]. Comparison of β-defensin-2 levels demonstrates significantly increased levels of β-defensin-2 expressed as ng per μg protein in the lesional AD skin (n=30~43) and in psoriatic skin (n=4) compared with the HC skin (n=26). On the other hand, there is no significant difference between HC skin and nonlesional AD skin. The content of β-defensin-2 in the lesional AD skin occurs at a significantly higher level than in the nonlesional AD skin, which indicates that inflammation is an essential factor that stimulates the production of β-defensin-2 in the epidermis of AD patients. When AD skin is classified according to different colony numbers, there is a weak correlation between increased levels of β-defensin-2 and increased numbers of *S. aureus* on the skin surface. Since there is a weak but significant positive correlation between the *S. aureus* colonization and the β-defensin-2 level in the lesional and nonlesional skin of AD patients, it is likely that β-defensin-2 is induced in response to bacteria, injury or inflammatory stimuli and is not associated with vulnerability to *S. aureus* colonization in the AD skin.When the relationship between the increased colonization and the levels of the anti-microbial peptide β-defensin-2 was assessed in the lesional and nonlesional skin of the same AD patients, there is a significant correlation (r=0.342, p=0.0046, n=67) between those two parameters, which indicates the possibility that the colonization of AD skin by *S. aureus* directly triggers the increased level of β-defensin-2 because it is known that *S. aureus* induces the expression of β-defensin. Based on the above results, as depicted in Figure 5, the colonization of *S. aureus* in AD skin may result from a decrease in the anti-microbial lipid, sphingosine, but not the anti-microbial peptide β-defensin-2 due to the ceramide deficiency which is deeply associated with the barrier disruption.

Is the Ceramide Deficiency Inherent or not?

In adult AD skin, there is a ceramide deficiency even in the

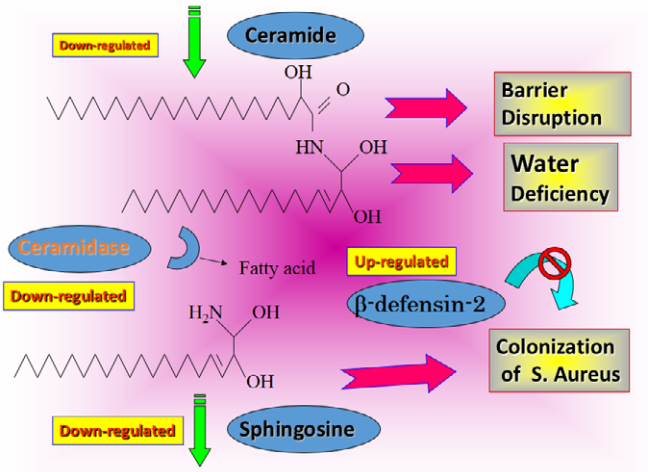

Figure 5: Possible Mechanism Leading to the Colonization of S. aureus [3,4].

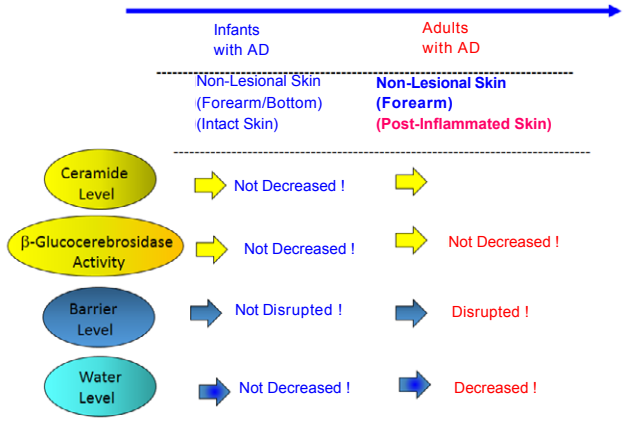

Table 2: Comparison of the levels of ceramide, BGCase activity, barrier function and water content between infants and adults with AD [8].

nonlesional SC, which is highly associated with the abnormal barrier function, and predisposes the skin to inflammatory processes evoked by irritants and allergens. Since adult AD nonlesional skin may be post-inflamed, it remains unclear whether the nonlesional barrier disruption and ceramide deficiency results from post-inflammation or from an inherited ceramide synthesis abnormality. To test this possibility, we used infants with AD to examine SC function and to compare SC ceramide levels and BGCase activity in their arm and buttock skin [8]. The TEWL in the forearm skin of infants with AD (n=24~26) was significantly up-regulated in the lesional skin but not in the nonlesional skin compared with HC skin (n=18~20). When the relationship between TEWL and scorad index in the lesional and nonlesional AD infant skin was assessed, there was a significant correlation between the two parameters in the lesional skin but not in the nonlesional skin, which suggests that the barrier disruption is not inherent but results from a subsequently elicited dermatitis. In contrast, the water content, as assessed by capacitance, was slightly but not significantly decreased only in the lesional arm skin. The ceramide levels, expressed as μg ceramide/mg protein, demonstrated that there is a significant decrease in SC ceramide in the lesional forearm skin (n=22) but not in the nonlesional forearm skin (n=28), which is consistent with the increased TEWL values only in the lesional skin. When BGCase activity in the SC was evaluated, there was no significant difference between HC skin (n=18~20) and AD lesional and nonlesional skin. In the buttock skin of infants with AD (n=26), there was no significant increase or decrease in TEWL values and water content, respectively, compared with HC skin (n=18). The ceramide levels, expressed as μg ceramide/mg protein, revealed that there is no significant decrease in SC ceramide in the nonlesional buttock skin which is consistent with the lack of an increase in TEWL values in the nonlesional skin. BGCase activity in the SC of the buttock skin demonstrated that there is no significant difference between the skin of HCs and AD infants.

In summary, as shown in Table 2, in the infant nonlesional AD skin, TEWL and water content was not altered compared with HC infant skin. Comparison of ceramide levels demonstrates that SC ceramides are significantly reduced only in the lesional forearm skin but not in the nonlesional skin of AD infants compared with HC infant skin. On the other hand, there was no significant difference in BGCase activity in the SC of the forearm and buttock skin between infant AD and infant HC skin as well as between adult AD and adult HC skin. These findings suggest that the barrier disruption due to the ceramide deficiency is not inherent and is essentially dependent on post-inflammatory events in infants with AD. These findings can reflect the atopic diathesis as a genotypic predisposition but are not consistent with loss-of function mutations in the FLG gene in AD which may represent only one facet of the atopic abnormalities.

Inflammation as a causative factor that down-regulates ceramide generation

Thus, the evidence for the involvement of inflammation in the predisposition to the ceramide deficiency which results in the impaired barrier function prompted us to determine the effects of evoked inflammation on barrier function and ceramide biosynthesis in the SC of AD patients. We used tape-stripping as an inducer of cutaneous acute inflammation to compare changes in the levels of barrier disruption and water content as well as ceramides and sphingolipid enzyme activities between AD skin (n=38) and HC skin (n=38) during the barrier recovery process [64]. A similar approach with tape-stripping was used for Nieman Pick patients, which showed that the delayed barrier recovery is mainly ascribed to the inherently down-regulated levels of SMase activity [65]. Based on this evidence, if the alteration of some ceramide-metabolic enzymes or their associated enzymes are involved as causative factors in the continued barrier disruption of AD skin, this approach would provide a deep insight into skin phenotypic changes in barrier function and ceramide content in the SC of AD patients in response to cutaneous inflammation as well as into unknown atopic diathetic factors which may be provoked following acute inflammation. The results showed that basal levels of skin capacitance values prior to the tape-stripping are significantly lower in patients with AD than HCs. During the tape-stripping process, the water content (capacitance value) increased with the increased number of tape-strippings to a lower extent in AD skin than in HC skin. During the recovery process, at 4 days post-tape-stripping, capacitance values in AD skin returned to almost the same values as before the tape-stripping. In contrast, those values in HC skin return to less than the pre-tape-stripping level. Thus, skin capacitance values occur at similar levels between AD and HC skin at 4 days post-tape-stripping. On the other hand, basal levels of TEWL values prior to the tape-stripping were significantly higher in

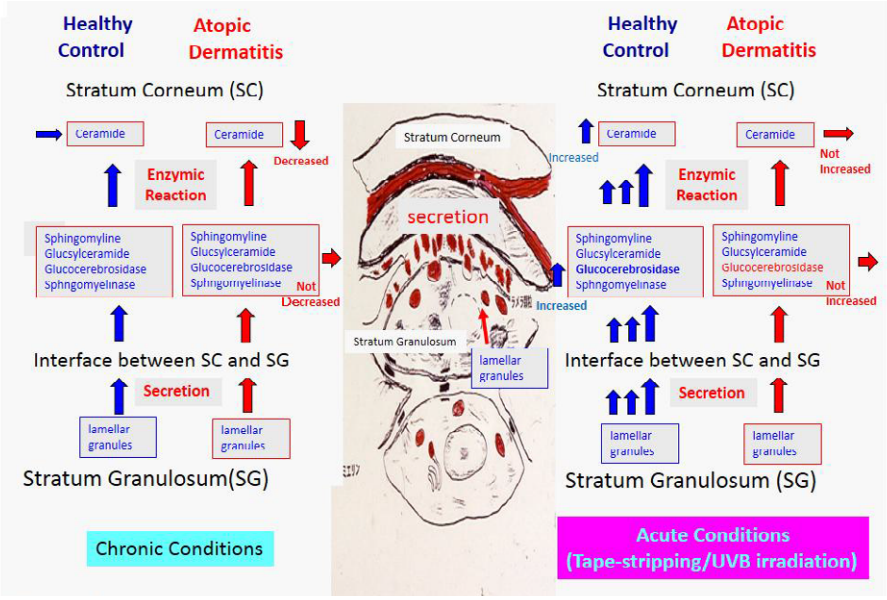

Figure 6: Summary of the Study using Tape-Stripping to Compare Ceramide Levels and Enzymatic Activities between Casual Chronic Conditions and Acute Conditions [65].

AD subjects than in HCs. During the tape-stripping process, TEWL values rose with the increasing number of tape-strippings to a similar extent in AD skin and HC skin. During the recovery process, at 4 days post-tape-stripping, TEWL values in both AD and HC skin returned to higher levels than before the tape-stripping, the level of which was significantly higher in AD skin than in HC skin. The recovery of TEWL values after tape-stripping occurred at a significantly slower rate at 1 day post-tape-stripping in AD skin than in HC skin, but proceeded at a similar rate at 2, 3 and 4 days post-tape-stripping. Similar recovery data were observed in Niemann-Pick disease with the SMase deficiency with a delay of barrier recovery only at 1 day post-tape-stripping [65]. Thin layer chromatographic analysis demonstrated that basal levels of ceramides in the SC significantly differ between AD subjects and HCs with significantly lower levels in AD skin (n=27) than in HC skin (n=17). Comparison of ceramide levels in the SC between before and after tape-stripping revealed that whereas ceramide levels in HC skin (n=17) are significantly up-regulated at 4 days post-tape-stripping, the ceramide levels in AD skin (n=27) are not substantially changed at 4 days post-tape-stripping. We have already reported that there are no significant differences in the activities of BGCase and SMase in the epidermis or the SC of AD patients and age-matched HCs [66,67]. CDase activity is rather down-regulated in the epidermis of AD all of which cannot account for the ceramide deficiency. Further, there have been no reports demonstrating any abnormalities of other sphingolipid metabolic enzymes, such as SPT, ceramide synthase or glucosylceramide synthase (glucosyl-transferase) in AD [67].

Analysis of ceramide species indicates that basal levels of Cers [NP], [AS], [NH], [AP] and [AH] are significantly lower in AD skin than in HC skin. Comparison of ceramide species before and after tape-stripping reveals that levels of Cers [EOS], [NP], [AP] and [AH] are significantly increased at 4 days post-tape-stripping in HC skin whereas levels of all ceramide species are unchanged in AD skin. When BGCase activity was assessed, basal levels of the enzyme activity in the SC occurred at a similar level between AD skin (n=20) and HC skin (n=18). Comparison of the enzyme activity in the SC before and after tape-stripping demonstrated that whereas the activities at 4 days post-tape-stripping were significantly up-regulated in HC skin (n=18) compared with before tape-stripping, those activities in AD skin (n=20) remained substantially unchanged. While BGCase activity shows more than a 144% increase in HC skin at 4 days post-tape-stripping, those activities are not up-regulated in AD skin. Because there is no significant difference in the basal levels of BGCase activity between HC skin and AD nonlesional and lesional skin, this strongly suggests that the failure to stimulate BGCase activity does not depend on any defect(s) specific for BGCase biosynthesis but is mainly attributable to an abnormality of the tape-stripping-induced secretion of lamellar granules through which levels of ceramide-precursors, SM as well as GCer, are also not accentuated concomitant with the non-stimulated protein level of BGCase. Whereas a possible abnormality of lamellar granule secretion in acute inflammatory conditions of AD may explain the continued ceramide deficiency, it cannot account for the lack of altered BGCase activity as well as SMase activity in both the lesional and nonlesional AD skin. Figure 6 shows a summary of the study using tape-stripping which compares ceramide levels and enzymatic activities between casual chronic conditions and acute conditions such as tape-stripped skin. Based on the fact that the barrier recovery after tape-stripping is delayed, as seen with SMase-deficient Niemann-Pick disease, it is possible that the impaired homeostasis of a ceramide-generating process other than BGCase, SMase and/or CDase may be associated with the continued abnormality of barrier and water reservoir functions due to the ceramide deficiency in nonlesional AD skin.

Biological Mechanisms Underlying the Ceramide Deficiency

It is feasible to speculate that the impaired barrier function of the SC in AD skin results from the decreased production of ceramides, which are generated from SM and GCer via the enzymatic reactions of SMase and BGCase, respectively, in the interface between the stratum

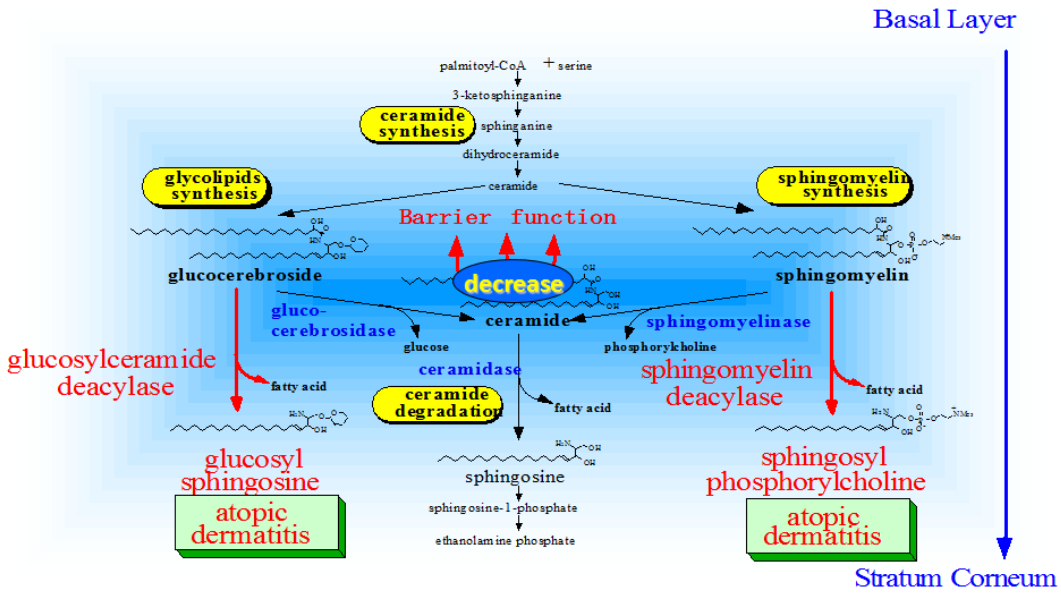

Figure 7: Possible Biological Mechanisms Involved in the Ceramide Deficiency of AD [14, 68-71].

granulosum and the SC. Nevertheless, there is no abnormality in the activities of either of those enzymes or the ceramide-degradative enzyme, CDase in the AD epidermis, so reductions in their activities cannot account for the ceramide deficiency [66-68]. Further, there have been no reports demonstrating any abnormalities of other sphingolipid metabolic enzymes, such as SPT, ceramide synthase or glucosylceramide synthase in AD. While elucidating the biological mechanisms by which the ceramide deficiency is induced but is not concomitant with decreased levels of BGCase, SMase or other sphingolipid metabolic enzymes, we discovered a novel sphingolipid metabolic enzyme termed sphingomyelin (SM)/glucosyceramide (GCer) deacylase, which cleaves the N-acyl linkage of SM and GCer [14,67-70]. This enzyme competes with BGCase and SMase to hydrolyze common substrates, SM or GCer, to yield sphingosylphosphorylcholine (SPC) or glucosylsphingosine (GSP), respectively, instead of ceramide, which results in the ceramide deficiency in AD. Analytical isoelectric focusing chromatography using the atopic SC demonstrated that the pI values of SM deacylase, SMase, BGCase and CDase are 4.2, 7.0, 7.4 and 5.7, respectively. These results indicate that a novel epidermal enzyme, SM deacylase, exists in the atopic SC with an enzymatic property distinct from other sphingolipid metabolic enzymes. In studies using [palmitic acid-1-^{14}C] SM as a substrate, a pH dependency of catalytic activity with a peak at pH 5.0 is shown. The gel chromatographic pattern of SM deacylase activity in the atopic SC showed that the apparent molecular mass of SM deacylase is 40,000. Finally, SDS-PAGE analysis revealed that the molecular weight of SM deacylase in the epidermis is approximately 42,000 while that of SMase is approximatly 100,000.

The question of whether CDase (N-acylsphingosine deacylase) is involved in the hydrolysis of the N-acyl linkage of SM remains to be elucidated. When SM deacylase of the SC from AD patients was partially purified by chromatography and assessed at pH 4.7 using [1-^{14}C] palmitoylsphingosine as a substrate, negligible levels of CDase activity were found in the partially purified fractions, which contained significant levels of SM deacylase activity [68]. Further, the 4.2 pI fraction, which is rich in SM deacylase activity, had no capacity to hydrolyze ceramide even under conditions where SM and GCer can be hydrolyzed [68]. Therefore, it is unlikely that CDase is involved in the activity of SM deacylase in the SC of AD patients. These enzymatic properties of SM/GCer deacylases expressed in the epidermis of AD patients indicate that they are novel epidermal enzymes distinct from any known N-deacylases or CDase.

Assays for SM deacylase, using fluorescent SM or radiolabeled palmitoyl SM as a substrate demonstrated that the SC from the lesional forearm skin of AD patients (n=13) has an extremely high level of the activity with a magnitude 5 times higher than in HC skin (n=20) [67]. In the nonlesional AD skin (n=14), the activity is still 3 times higher than in HC skin. In contrast, the involved SC from contact dermatitis patients shows levels similar to HC skin. Very importantly, in the germ free-lesional epidermis of AD patients, the activity is also significantly up-regulated by 3-fold over HC skin, which eliminates the concern that the SM deacylase is derived from skin bacteria [67]. Assays for GCer deacylase using palmitoyl ^{14}C-GCer as a substrate revealed that the SC from the lesional forearm skin of AD patients (n=19) has an extremely high level of activity with a magnitude 6 times higher than in HC skin (n=15). In the nonlesional AD skin (n=19), this activity is still 3 times higher than in HC skin [14]. In contrast, there are no significant differences in BGCase activity between the SC of AD skin and HC skin. On the other hand, the involved SC from patients with chronic eczema shows levels similar in both enzyme activities to HC skin. These comparative data with other types of dermatitis are very important to elucidate factors specific for the atopic phenotype because many similar studies have just compared AD only with HC skin. Consistent with the high expression of SM deacylase in AD skin, quantitative analysis of its metabolite, SPC, per mg SC using a radiolabeling method similar to that used for sphingosine measurement reveals that there is a significant increase in SPC in both the uninvolved and involved SC (n=44~47) compared with age-matched HC skin (n=40) [15]. In contrast, there is no increase in the SPC content in the involved SC of patients with chronic eczema (n=6). Consistent with the high expression of GCer deacylase, quantitative analysis of its metabolite, GSP per mg SC using a

radiolabeling method similar to that used for sphingosine measurement shows that there is a significant increase in GSP in both the uninvolved and involved SC from AD patients (n=94~105) compared with age-matched HC skin (n=81) [14]. In contrast, there is no increase in GSP in the involved SC of patients with chronic eczema (n=7). Our hypothesis for the mechanism underlying the ceramide deficiency of AD skin is shown in Figure 7, in which novel SM/GCer deacylases are expressed in the epidermis of AD patients and these enzymes utilize SM or GCer as a substrate to yield SPC or GSP instead of ceramide, leading in turn to the ceramide deficiency.

Regarding the biochemical connection and clinical loop among the ceramide deficiency, barrier disruption, altered sphingolipid metabolism and atopic phenotypes, we have already found that SPC, a metabolite of the enzymatic action of SM/GCer deacylase, induces the expression of intercellular adhesion molecule-1 expression and transglutaminase activation in keratinocytes and accelerates melanin synthesis in melanocytes [71-73]. These findings indicate that atopic phenotype such as inflammation, roughened skin and hyperpigmentation, are in part associated with the altered sphingolipid metabolism.

As depicted in Figure 8, a similar accumulation of reaction products by corresponding N-deacylase enzymes has been found in Gaucher disease Niemann-Pick disease and Krabbe's disease in which there is an accumulation of GSP, SPC and psychosine as a result of a defect of BGCase, SMase and galactosylceramidase activities, respectively [74-76]. Such altered lipid metabolisms associated with genetic defects, which lead to the accumulation of lipid substrates and deacylated metabolic intermediates, strongly suggest the principle that defects of metabolic enzymes might induce corresponding alternative pathways in which those substrates are converted to corresponding lysoforms by deacylation. Such a possible induction of an alternative pathway after the loss of metabolic enzymes has been reported in a Gaucher-like mouse induced by a glucosylceramidase inhibitor that shows the accumulation of GS in tissue [76]. Such a similarity in the accumulation of lysosphingolipids as bioproducts by N-deacylases between AD and Gaucher or Niemann-Pick diseases seems to suggest the possibility that in AD skin, unveiled biological influences, such as the deterioration of SMase or BGCase enzymes, may induce the expression of such bypass enzymes under the unknown conditions associated with the atopic diathesis.

Recently, SM deacylase has been purified as a 40 kDa protein to homogeneity from rat skin, which allowed its amino acid sequence to be determined and then identified as the β-subunit of CDase, which consists of α- and β-subunits linked by two S-S bonds [77]. In fact, breaking the two S-S bonds of recombinant CDase by dithiothreitol elicited the activity of SM deacylase. Although the highly elevated activity of SM deacylase in AD skin seems to play a key role in the ceramide deficiency, it remains unclear why the β-subunit of CDase is released at the interface between the SC and stratum granulosum layers in AD skin. As depicted in Figure 9, we hypothesized two possible mechanisms underlying the expression of SM/GCer deacylase as follows: 1) S-S crosslinking enzymes which bind the α- and β subunits of CDase may be down-regulated or malfunction in AD skin; 2) S-S breaking enzymes which release the β-subunit from CDase are up-regulated or over-expressed in AD skin.

Clinical Efficacy of Ceramide as a Major Factor in the Pathogenesis of AD

Finally, to provide a deeper insight into the impaired barrier mechanisms which are associated with either ceramide or filaggrin-derived water-soluble materials (mainly amino acids), it was of considerable interest to determine whether compensating either ceramides or water-soluble materials would replenish the barrier function in clinically normal, nonlesional AD skin. This approach seems to be a key for preventing the refractory nature of the dermatitis and provides a mechanistic insight into the pathogenesis of AD. We

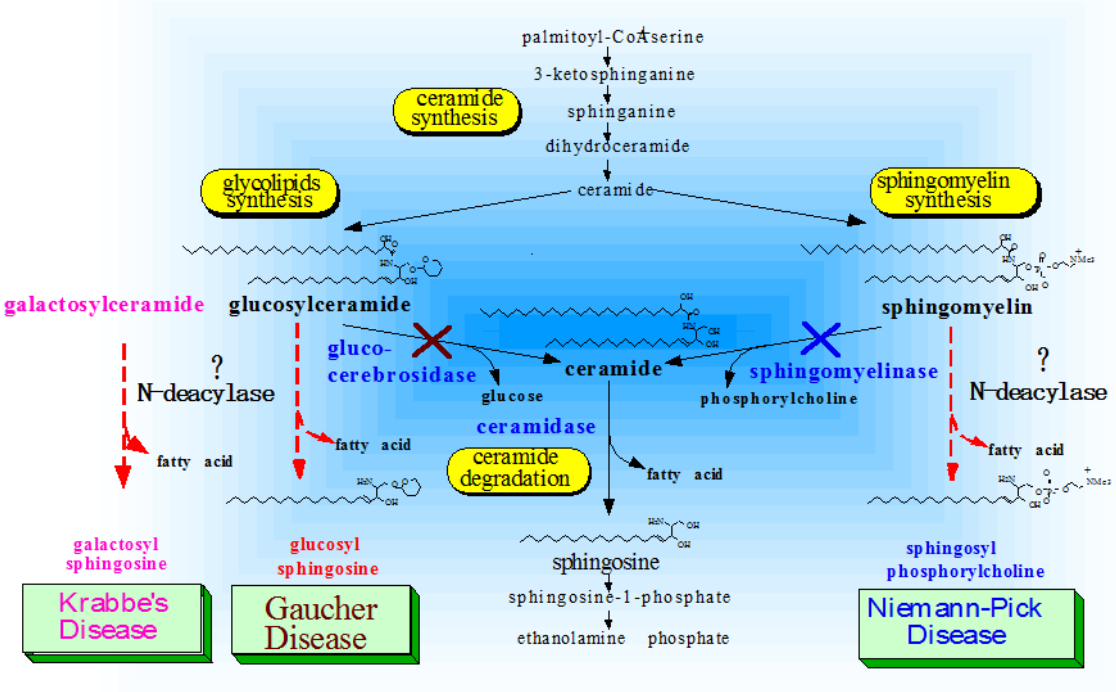

Figure 8: Itered Sphingolipid Metabolisms Discovered in Gaucher, Niemann-Pick and Krabbe's Diseases [75-77].

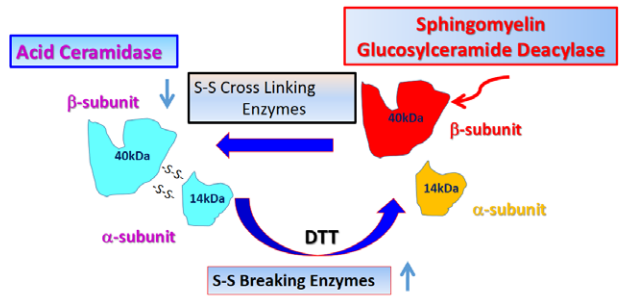

Figure 9: Possible Mechanisms Involved in the Expression of Sphingomyelin Glucosylceramide Deacylase [78].

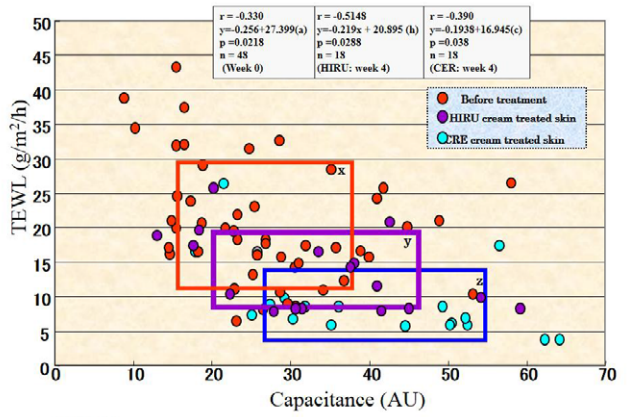

Figure 10: Comparison between TEWL and Capacitance Values during 4 Weeks of Treatment with CER or HIRU Creams in Association with Disease Severity [80].

performed a clinical comparison in nonlesional right or left forearm AD skin (n=24) treated for 4 weeks with 8% synthetic pseudo-ceramide (CER) cream or 0.3% mucopolysaccaride (HIRU) cream, the latter of which is used as a medical moisturizing cream for AD in Japan [78]. Comparison of the two creams for clinical improvement reveals that whereas the HIRU cream resulted in 80% of AD subjects having a slight improvement, and 20% having a moderate improvement, the CER cream resulted in 50% of AD subjects having a marked improvement, 36% having a moderate improvement and 15% having a slight improvement, which was significantly more efficient compared with the HIRU cream. The CER cream induced a significant reduction in TEWL values with a significant and sharp decrease appearing at weeks 2 and 4. In contrast, the HIRU cream elicited a significant but lesser reduction in TEWL values at weeks 2 and 4 than did the CER cream. A comparison of the reduced TEWL values demonstrates that treatment with the CER cream elicited a significantly greater reduction in TEWL ($p<0.0001$) at week 4 compared with the HIRU cream. The CER cream also induced a significant increase in capacitance values at weeks 2 and 4. In contrast, the HIRU cream elicited a significant but lesser increase in capacitance values only at week 2 than did the CER cream. A comparison of the increased capacitance values demonstrates that at weeks 2 and 4, treatment with the CER cream elicited a significantly greater increase in capacitance values compared with the HIRU cream. As previously described in Figure 6, when TEWL and capacitance in association with disease severity was compared, rectangular areas representing the TEWL and capacitance values are well distributed in association with severe, moderate and mild groups of AD and HCs [6]. As shown in Figure 10, comparison between TEWL and capacitance values at 4 weeks of treatment in association with disease severity reveals that whereas those two parameters of the CER cream treated skin are generally distributed in the area corresponding to the HC, those values for the HIRU cream treated skin remained within the area corresponding to the mild or moderate group of AD, indicating the superior clinical efficacy of the CER cream. In another clinical study of AD patients (n=40) treatment for 4 weeks with the CER cream significantly reduced the dryness/scaling/itchiness, which was accompanied by significant decreases in TEWL and increases in capacitance values as well as by down-regulated nicotinate sensitivity at 4 weeks [79]. The efficacy of the CER cream was also evidenced by the facts that there was a close correlation between the level of CER that penetrated into the SC and the increased capacitance values, and that the ceramide profiles, including their shorter alkyl chain lengths, distinctly changed from an atopic to a healthy phenotype at 4 weeks. These results suggest an inflammation but not an atopy-specific phenotype of ceramide alteration. Taken together, the clinical efficacy of synthetic CERs, focusing on the barrier/water replenishment and the ceramide profile, provides a deep insight into the pathogenesis of AD as a ceramide-deficient disease in which barrier/water function is markedly attenuated and predisposes the skin to cutaneous inflammation that elicits an abnormality in the ceramide profile including a shorter alkyl chain length. The efficacy for the barrier recovery following the ceramide treatment was also evidenced by the fact that, while MAs applied under occlusive conditions can easily penetrate the dermis of the clinically normal skin of AD patients, the pre-application of synthetic pseudo-ceramides including pseudo-acylceramides abolished the MA penetration, resulting in a marked reduction of MA-dependent cutaneous allergic reactions compared with untreated skin [20,80]. Taken together with the close relationship between atopic phenotypes and barrier function, the observed clinical efficacy using the synthetic pseudo-ceramide provides a deep insight into the pathogenesis of AD as a ceramide-deficient barrier disease.

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