Ceramide synthase 4 is highly expressed in involved skin of patients with atopic dermatitis

Running Head: CERS4 in epidermis with atopic dermatitis.

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Abstract

Background Ceramide is a crucial lipid in the stratum corneum (SC), which maintains the barrier function and hydration of the skin. In atopic dermatitis (AD) patients who have defective skin barrier function, ceramide levels are altered. We previously reported that although the amount of total ceramide was lower in involved skin compared with uninvolved skin of AD patients and with healthy control skin, the amounts of smaller ceramide species (<40 total carbons) of Cer[NS], especially Cer[NS] with 34 total carbons (C34-Cer[NS]), were higher. However, the enzyme(s) that produces the higher levels of smaller ceramide species in involved skin of AD patients was unclear.

Objective To identify the enzyme(s) that produces higher levels of smaller ceramide species of Cer[NS] in the involved skin of AD patients.

Methods Eight female Caucasian subjects who were diagnosed with AD on their arms (age range: 21-45 yrs.) were enrolled in this study. We compared ceramide levels in the SC, and the expression levels of enzymes involved in ceramide metabolism using real-time PCR and immunohistochemistry between involved and uninvolved skin of AD patients.

Results Level of mRNA encoding ceramide synthase 4 (CERS4), which is one of the enzymes that synthesize ceramide from a sphingoid base and an amide-linked fatty acid, were

significantly higher in involved skin than in uninvolved skin ($p<0.01$). Additionally, the protein expression level of CERS4 in the epidermis was also higher in involved skin compared with uninvolved skin. The expression level of CERS4 correlated with the amount of C34-Cer[NS] ($p<0.01$) and the skin hydration value ($p<0.05$).

Conclusions The elevated expression level of CERS4 contributes to the increase of C34-Cer[NS] and the impaired SC barrier function in involved skin of AD patients.

Abbreviations

SC, stratum corneum; Cer, ceramide; AD, atopic dermatitis; TEWL, transepidermal water loss; CerS, ceramide synthase

Keywords

atopic dermatitis; ceramide; ceramide synthase

Introduction

Intercellular lipids in the stratum corneum (SC) play an important role in the permeability barrier function and water-holding capacity of the skin.[1] Intercellular lipids in the SC mainly consist of

ceramide (Cer), cholesterol and free fatty acid. Ceramide, which is the main component, is essential for the barrier function and skin hydration.[2] In the SC of atopic dermatitis (AD) patients, who have an impaired barrier function, the levels of total ceramide are decreased compared to healthy controls, especially in involved skin.[3-5] In addition, in AD patients, the amount of ceramide in the SC correlates with two characteristic measures of skin barrier function, transepidermal water loss (TEWL) and capacitance value.

Twelve major ceramide classes (Cer[NDS], Cer[NS], Cer[NH], Cer[NP], Cer[ADS], Cer[AS], Cer[AH], Cer[AP], Cer[EODS], Cer[EOS], Cer[EOH] and Cer[EOP]) have been identified in the SC of human skin.[6-8] Each ceramide class contains ceramide species with different carbon chain lengths. Interestingly, even though the total amounts of Cer[NS] are not changed in involved skin of AD patients, the average number of total carbon atoms in Cer[NS] is altered. Specifically, Cer[NS], which contains a long carbon chain length (>50 total carbons), is low, whereas Cer[NS], which contains a short carbon chain length (<40 total carbons), is high in involved AD skin compared with uninvolved skin and with healthy controls. That tendency is also evident in Cer[NDS]. It should be noted that not all ceramide species are decreased, and some ceramide species, for instance smaller species of Cer[NS] (<40 total carbons), are increased in AD patients, especially in involved skin. In addition, the amount of Cer[NS] with 34 total carbons

(C34-Cer[NS]) is strongly correlated with the impaired SC functions (higher TEWL and lower capacitance).[9,10] Hence, the increased levels of smaller species of Cer[NS] in the SC are related to impaired barrier function. However, the reason for the increased amounts of smaller species of Cer[NS] was unresolved.

Ceramide is generated from a sphingoid base and an amide-linked fatty acid by ceramide synthase (CerS).[11] It was reported that 6 CerS family members (CerS1-6) are expressed in mammalian cells and 5 types of CERS (CERS2-6) are expressed in human keratinocytes.[11] Each CerS member exhibits a preference for a specific carbon chain length of fatty acyl-CoAs as substrate.[12,13] Thus, we hypothesized that one or more CerSs are involved in the increase of smaller species of Cer[NS].

In this study, to clarify changes in enzyme(s) potentially responsible for the increase of smaller species of Cer[NS], especially C34-Cer[NS], in involved skin of AD patients, we compared the gene expression levels of CerSs and the distribution of protein expression in the epidermis between involved and uninvolved skin of AD patients.

Materials and Methods

Subjects

Eight subjects who were diagnosed with AD on their arms (Caucasian, female, age range: 21-45 yrs; mean 32.7 yrs) were enrolled in this study. The study was conducted according to the Declaration of Helsinki Principles. The protocol was approved by the Institutional Review Board of IntegReview Ltd. (Austin, TX) and the Ethics Committees of the Kao Corporation. Informed consent to participate in this study was obtained from all subjects.

TEWL and skin hydration

The arm of each AD patient that included involved and uninvolved skin was washed with soap (Kao Corporation, Tokyo, Japan) before measurements. After 20 min of acclimation, TEWL and capacitance were measured using a Tewameter TM300 and a Corneometer CM825 (Courage+Khazaka Electronic GmbH, Cologne, Germany), respectively, on the involved and uninvolved skin on the arm of each AD patient.

Extraction of ceramides from the stratum corneum

SC specimens from involved and from uninvolved skin of each AD patient was assessed by tape-stripping as follows: A polyphenylene sulfide film tape (Nichiban, Tokyo, Japan) with an area of 25 mm × 30 mm was pressed on the targeted area and then was removed. Three or 5

consecutive tapes were collected from that same area on each patient. The SC-stripped tapes were stored at -20°C until extraction of ceramide, which was performed as described in previous reports.[7, 8] In brief, each SC-stripped tape was cut into two equal parts. One part was used to prepare extracted lipid samples and the other part was used for the analysis of protein amounts. The tape strips were immersed in methanol and were then sonicated. After the lipid extracts were dried, they were dissolved in chloroform/methanol (99.5:0.5, by vol). This lipid solution was applied to a Sep-Pak Vac RC silica cartridge (Waters, Milford, MA) that had been conditioned with chloroform/methanol (99.5:0.5, by vol), followed by solid phase extraction with 10 ml chloroform/methanol (99.5:0.5, by vol) and 10 ml chloroform/methanol (99:5, by vol). After the latter fraction was dried, it was dissolved in n-hexane/2-propanol/formic acid (95:5:0.1, by vol).

Determination of soluble proteins

The rest of the tape pieces were immersed into 0.1 M sodium hydroxide containing 1% (w/v) sodium dodecyl sulfate aqueous solution and were then incubated at 60°C for 2 hours to obtain soluble proteins. After incubation, the solution was neutralized with 2 M hydrochloric acid aqueous solution. Soluble protein amounts were detected using a BCA protein assay kit (Pierce, Rockford, IL).

Ceramide analysis using LC-ESI-MS

Ceramides were analyzed using an Agilent 1100 Series LC/MSD single-quadrupole system equipped with an electrospray ionization source, ChemStation software, an 1100 well plate autosampler (Agilent Technologies, Palo Alto, CA) and an Inertsil SIL 100A-3, 1.5 mm i.d. × 150 mm column (GL Science, Tokyo, Japan). These procedures are detailed in our previous reports.[7,8]

Preparation of epidermal sheets

Four mm punch biopsies were obtained from involved and from uninvolved skin of each AD subject. The epidermal sheets of those skin biopsies were peeled from the dermis after overnight incubation in dispase solution (BD Biosciences, Franklin Lakes, NJ) with Epi-life medium (Invitrogen, San Diego, CA) (1:15, by vol) at 4°C, after which the epidermal sheets were stored in RNAlater (Qiagen, Valencia, CA) solution at -80°C until extraction.

Real time PCR

The epidermal sheets were homogenized by Tissue-Tearor (Biospec Products Inc, Bartlesville,

OK). Total RNAs were isolated using an RNeasy Micro Kit (Qiagen) and cDNAs were synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturers' protocols. Real time quantitative RT-PCR was performed with the TaqMan Gene Expression Assay (Applied Biosystems), and on-demand probes for each target gene, Hs00371958_g1; ceramide synthase 2 (CERS2), Hs00226114_m1; ceramide synthase 4 (CERS4), Hs00332291_m1; ceramide synthase 5 (CERS5), Hs00826756_m1; ceramide synthase 6 (CER6) (Applied Biosystems). Results were normalized against RPLP0 (ribosomal protein large P0, Hs99999902_m1, Applied Biosystems) using an ABI PRISM 7300 Sequence Detector System (Applied Biosystems).

Immunohistochemistry

Punch biopsy specimens were cut into pieces and embedded in OCT freezing compound (Sakura Tissue Tek, Tokyo, Japan). The specimens were frozen with isopentene pre-cooled using liquid nitrogen. The OCT blocks were cryosectioned into 6-μm thick sections and were kept at -80°C. For immunohistochemistry, the frozen sections were fixed with pre-cooled acetone at -20°C. After the acetone evaporated, the sections were equilibrated with PBS and treated with 0.1% Triton X-100 in PBS, followed by washing in PBS and blocking of endogenous peroxidase

activity with 0.3% hydrogen peroxide solution. The sections were then blocked with Ultra V Block (Thermo Fisher Scientific, Waltham, MA). After incubation with the primary antibody (anti-CerS4, sc-65112 (Santa Cruz Biotechnology, Santa Cruz, CA), a biotinylated rabbit anti-goat (Dako, Denmark) secondary antibody was applied to the sections. For visualization, HRP-linked streptavidin and AEC substrate (both from Thermo Fisher Scientific) were used. The sections were then counterstained with hematoxylin (Thermo Fisher Scientific).

Statistics

Data were analyzed for statistical significance using the paired t-test. The difference between involved and uninvolved skin is considered statistically significant when $p < 0.05$. The relationship among gene expression level, amounts of ceramide and SC functional parameters were analyzed using Pearson's correlation coefficient.

Results

TEWL and Capacitance are changed in involved skin compared to uninvolved skin of AD patients

Consistent with prior reports [9 14], the TEWL values of involved AD skin were almost four times

higher than in uninvolved skin ($p<0.05$, n=8) (Fig. 1a). Furthermore, the capacitance values of involved skin were approximately half of the values of uninvolved skin ($p<0.01$, n=8) (Fig. 1b). Both of these findings are consistent with the known alterations of barrier function in the skin of AD patients.

Changes of detailed ceramide composition in involved skin of AD patients

The total ceramide amounts in involved skin were also nearly half the levels in uninvolved skin ($p<0.01$, n=8) (Fig. 2a). Among the 11 ceramide classes investigated, the amounts of Cer[NDS], Cer[NH], Cer[NP], Cer[ADS], Cer[AH], Cer[AP], Cer[EOS], Cer[EOH] and Cer[EOP] were significantly lower in involved skin compared with uninvolved skin ($p<0.01$, n=8) (Fig. 2b). Additionally, the average numbers of total carbon atoms of Cer[NDS], Cer[NS], Cer[NH], Cer[AS] and Cer[AH] were significantly lower, but that of Cer[EOP] was significantly higher, in involved skin (data not shown). Major differences were found in Cer[NS] (Fig. 2c). In Cer[NS], the amounts of smaller species (number of total carbon atoms less than 39) in involved skin were significantly higher than in uninvolved skin, but the amounts of larger species (>46 total carbons) were significantly lower in involved skinthan in uninvolved skin ($p<0.05$ or $p<0.01$, n=8, respectively).

Ceramide synthase 4 is highly expressed in involved skin of AD patients

It has been reported that the gene expression level of CERS3 is not significantly changed between involved and uninvolved skin of AD patients.[15] Hence we analyzed the gene expression levels of CERS2, CERS4, CERS5 and CERS6. Analysis of the gene expression levels of those 4 CERSs revealed that only CERS4 was significantly changed, where it was significantly higher in involved skin than in uninvolved skin ($p<0.05$, $n=8$) (Fig. 3a). Next, we compared the protein expression pattern of CERS4 between the epidermis of involved and of uninvolved AD skin using immunohistochemistry. CERS4 was expressed from the suprabasal layer to the SC in uninvolved and in involved skin, however the staining intensity was obviously increased from the suprabasal layer to under the SC in involved skin (Fig. 3b).

Correlation between the expression level of CERS4 and the amounts of smaller ceramide species of Cer[NS] and SC function

We then assessed the relationship between the gene expression level of CERS4 and the amounts of smaller species of Cer[NS], which were decreased in involved skin of AD patients. From C33 to C39-Cer[NS], the amounts of C33-Cer[NS] and C34-Cer[NS] were significantly correlated

with the gene expression level of CERS4 ($r=0.560$, $p<0.05$ and $r=0.676$, $p<0.01$, respectively, Fig. 4a, 4b). Next, we assessed the correlation of the gene expression level of CERS4 and functional parameters of the SC, TEWL and capacitance values. A positive correlation between TEWL values and the gene expression level of CERS4 was observed ($r=0.461$, $p=0.07$, Fig. 4c), whereas a negative correlation between capacitance values and the gene expression level of CERS4 was observed ($r=-0.506$, $p<0.05$, Fig. 4d).

Discussion

The results of this study reveal that the gene expression level and protein expression of CERS4 is higher in involved AD skin than in uninvolved skin among the four CerSs examined (Fig. 3). In addition, the gene expression level of CERS4 is strongly correlated with C34-Cer[NS], which is remarkably increased in involved skin of AD patients (Fig. 2c and Fig. 4b). These results suggest that the high expression level of CERS4 is involved with the increased level of C34-Cer[NS] in involved skin of AD patients.

It has been reported that the sphingoid base in smaller species of Cer[NS] (less than 40 total carbons) is C16-C18.[7] Therefore, C34-Cer[NS] should consist of a C16:0 or C18:0 fatty acid. CerS5 and CerS6 mainly catalyze the synthesis of ceramide with a short fatty acid (C14:0 and

C16:0).[12] Nevertheless, the gene expression levels of CERS5 and CERS6 were not changed between involved and uninvolved skin (Fig. 3a). Otherwise, CerS2 and CerS4 can synthesize ceramide from C14:0, C16:0, C18:0, C20:0, C22:0,C24:0 and C26:0 fatty acyl-CoA as a substrate.[12] However, only the gene expression of CERS4 was significantly increased in involved skin of AD patients. Although CerS4 can synthesize ceramide with a C14-C26 fatty acid, the over-expression of CerS4 induced a remarkable increase of Cer[NS] with C16:0, C18:0 and C20:0 fatty acid in human embryonic kidney 293T cells.[16] In addition, among ceramide species with a C16-C26 fatty acid, only ceramide species which consist of C18:0 and C20:0 fatty acid were significantly decreased in the epidermis of CerS4-deficient mice.[17] These results indicate that although CerS4 has a potential to synthesize ceramide with a C14-C26 fatty acid *in vitro*, CerS4 plays a role in the synthesis of ceramide with C16:0, C18:0 and C20:0 in living organisms. In this study, we found that the expression level of CERS4 should contribute to the synthesis of C34-[NS]. Therefore, CERS4 may use C16:0 and C18:0 acyl-CoA as substrate to synthesize the high level of C34-Cer[NS] in involved skin of AD patients.

Fatty acid chain length has been reported to change in the SC of AD patients.[10] In particular, a short chain length fatty acid, especially C16:0 and C18:0, were significantly increased in involved skin of AD patients compared to uninvolved skin and normal control skin. In contrast,

more than C24:0 fatty acids were remarkably decreased in involved skin. Those composition changes should be related to the altered chain length of ceramide species. These results indicate that higher levels of C16:0 and C18:0 fatty acids and the high expression level of CERS4 cause the increase of C34-[NS] in involved skin of AD patients.

The alteration of chain length of fatty acids is effected by the expression and/or enzymatic activities of Elongases (ELOVL1-7).[18] Each ELOVL has a substrate specificity of fatty acid that depends on the chain length. Elovl4 is essential to the synthesis of long carbon chain length fatty acids and contributes to the synthesis of ceramide with chain lengths of more than C28, however, the amounts of ceramide with C16-C25 fatty acids were not remarkably changed in the epidermis of Elovl4-deficient mice.[19,20] Otherwise, Elovl1-deficient mice showed that the amounts of ceramide with chain lengths of more than C26 fatty acids were decreased whereas the amounts of ceramide with less than C24 were significantly increased.[21] Therefore, it may be suspected that the expression and/or enzymatic activity changes of ELOVL1 are involved with the increase in short chain fatty acid and contribute to the high levels of smaller species of Cer[NS] in the involved skin of AD patients.

The expression level of CERS4 correlated with SC functional parameters (Fig. 4c, 4d). This may be due to the increase of C34-Cer[NS]. Increasing ceramide levels with a short chain as well

as a short chain fatty acid leads to altered lipid organization in the SC.[22] In particular, that increases the hexagonal packing and disturbs the lamellar organization of the SC. These data suggest that high level of CERS4 may result in changing the lipid organization in the SC and may lead to the impaired barrier function in involved skin of AD patients.

To summarize, our results reveal that the high expression of CerS4 may be responsible, at least in part, for the increased content of Cer[NS] containing a short carbon chain length in involved AD skin, and as such, could contribute to the diminished barrier function in the skin of AD patients.

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