Treatment with Synthetic Pseudoceramide Improves Atopic Skin, Switching the Ceramide Profile to a Healthy Skin Phenotype

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Little is known about the pathophysiological linkages between altered ceramide profiles in the stratum corneum (SC) of patients with atopic dermatitis and their impaired skin barrier and water-holding functions. We studied those characteristics following topical treatment with a designed synthetic pseudoceramide (pCer) and analyzed that pathophysiological linkage by microanalyzing ceramides using normal phase liquid chromatography-electrospray ionization mass spectrometry. Four weeks of treatment with pCer significantly reduced skin symptoms, accompanied by significant decreases in transepidermal water loss and increases in water content. In the SC ceramide profiles, ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines (Cer[NH]) and ceramides containing nonhydroxy fatty acids and phytosphingosines (Cer[NP]) increased, whereas ceramides containing nonhydroxy fatty acids and sphingosines (Cer[NS]) and ceramides containing a-hydroxy fatty acids and sphingosines (Cer[AS]) decreased, with larger alkyl chain lengths in Cer[NS], distinctly representing a switch from an atopic dermatitis to a healthy skin phenotype. The level of pCer that penetrated into the SC was significantly correlated with the SC water content but not with transepidermal water loss. The level and the average chain length of Cer[NS] were closely correlated with the pCer level in the SC. These findings indicate that the penetrated pCer contributes to shift the ceramide profile from an atopic dermatitis to a healthy skin phenotype. Taken together, the observed clinical efficacy of treatment with pCer provides a deep insight into the pathogenesis of atopic dermatitis as a ceramide-deficient disease.

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INTRODUCTION

Atopic dermatitis (AD) is a recurrent dermatitis with a high susceptibility to itching, irritants, and allergens even in nonlesional skin, which is characterized clinically by severe dry skin and functionally by cutaneous barrier disruption and impaired water-holding function. Many studies have shown that the barrier-disrupted dry skin of patients with AD is mainly attributable to significantly decreased levels of ceramides in the stratum corneum (SC) (Arikawa et al., 2002; Choi and Maibach, 2005; Di Nardo et al., 1998; Imokawa et al., 1991a; Ishibashi et al., 2003; Ichikawa et al., 2010; Jungersted et al., 2010; Kim et al., 2017; Okamoto et al., 2003; Sugiyama et al., 2014). That mechanism is based on evidence that ceramide can function as a water reservoir (Imokawa and Hattori, 1985; Imokawa et al., 1991b) and also as a permeability barrier because of the formation of multilayered lamellar structures with other lipids, such as cholesterol ester and fatty acids, between the SC layers (Holleran et al., 1994a, 1994b, 1993, 1991a, 1991b). Further, the integrity of lipid lamellae in the SC of AD skin is distinctly diminished as a result of alterations in the ceramide profile, including the total ceramide level, its composite species, and its alkyl chain properties (Ishikawa et al., 2010; Janssen et al., 2011). Further, the disrupted barrier function and water deficiency that occurs in both the nonlesional and lesional skin of patients with AD (Matsuki et al., 2004a) and in essential fatty acid-deficient mice or is elicited by surfactant or solvent treatment of normal skin can be repaired by the topical application of natural ceramides (Imokawa, 2004; Imokawa et al., 1989c, 1986) or by synthetic pseudoceramides (pCer) (Funasaka et al., 2004; Hata et al., 2002b; Imokawa, 2004, 2001, 1999, 1994; Matsuki et al., 2004a; Mizutani et al., 2001; Nakamura et al., 2000; Takagi et al., 2004).
Role of Ceramide in Atopic Dermatitis

The goal of this study was to characterize the pathophysiological linkages between altered ceramide profiles in the SC of patients with AD and their impaired skin barrier and water-holding functions. We first determined the clinical and SC functional efficacy following topical treatment with a designed synthetic pCer and characterized that pathophysiological linkage by microanalysis of ceramides using normal-phase liquid chromatography—electrospray ionization mass spectrometry. The results of this study indicated that topical treatment with pCer significantly reduced skin symptoms, accompanied by significant decreases in transepidermal water loss (TEWL) and increases in water content. Those changes were associated with a distinct switch of the ceramide profile from an AD to a healthy skin phenotype without any increased level of endogenous natural ceramides. Taken together, the observed clinical efficacy of treatment with pCer provides a deep insight into the pathogenesis of AD as a ceramide-deficient disease.

RESULTS

Clinical efficacy of the pCer test lotion

The mean clinical symptom severity scores through the 4 weeks of the study are shown in Figure 1. All clinical scores tested, including erythema, scaling, lichenification, and excoriation, were significantly improved across all time points from 1 week through 4 weeks relative to the baseline at the onset of the clinical study using the pCer lotion (Figure 1a). Clinical evaluation of skin sensations revealed that sensation factors, such as stinging, burning, and itchiness, were significantly improved after 1 week of treatment with the pCer lotion and continued to improve significantly through the 4 weeks of treatment compared with the baseline (0 week) (Figure 1b and Supplementary Figure S1).

Changes in the water content and the water evaporation

Water content in the SC of lesional and nonlesional AD skin was measured by skin conductance and was markedly increased 1 day after treatment with the pCer lotion and remained at a significantly higher level during the 4 weeks of treatment with the pCer lotion and the 7 days of the regression phase (Figure 2a). TEWL values decreased significantly at week 2 and 4 during the 4 weeks of treatment with the pCer lotion and remained at a lower level at day 3 in the regression phase in the AD lesional skin (Figure 2b). This indicates that the water evaporation (inside-out barrier) was markedly improved by treatment with the pCer lotion in the AD lesional skin.

Changes in the levels of endogenous ceramide and penetrated pCer in the SC

Analysis of the ceramide content in the SC revealed that there was no increase in endogenous ceramide levels in the SC even after 4 weeks of treatment with the pCer lotion (Figure 2c). Quantitative analysis of the applied pCer revealed that the same average level of pCer as endogenous ceramides accumulated in the SC at week 4 of treatment (Figure 2c).
Changes in ceramide distributions before and after 4 weeks of treatment with the pCer lotion

The distribution profiles of ceramide species in the SC of nonlesional AD skin revealed that, whereas levels (by ng/mg protein) (Figure 3) or class ratios (by weight %) (Supplementary Figure S2) of Cer[NH], Cer[NP], ceramides containing a-hydroxy fatty acids and dihydrosphingosines (Cer[ADS]), ceramides containing a-hydroxy fatty acids and 6-hydroxysphingosines (Cer[AH]), and ceramides containing a-hydroxy fatty acids and phytosphingosines (Cer[API]) significantly increased after 4 weeks of treatment, those of Cer[NS]; Cer[AS]; ceramides containing ester-linked fatty

Changes in skin condition-associated factors and endogenous ceramide levels of patients with AD during 4 weeks of treatment with the pCer lotion

(a) Changes in water content (conductance) of the SC of lesional and nonlesional AD skin during 4 weeks of treatment with the pCer lotion and in the 7 days of regression phase. n = 38, data represent mean ± SD. (b) Changes in the barrier function (TEWL) of the SC of lesional and nonlesional AD skin during 4 weeks of treatment with the pCer lotion and in the 7 days of regression phase. n = 38, data represents mean ± SD. (c) Endogenous ceramide levels in the SC and pCer that penetrated into the SC in the nonlesional AD skin on the volar forearm before and after 4 weeks of treatment with the pCer lotion. n = 38, data represent individual values and mean ± SD. *P < 0.05 and **P < 0.01 versus baseline; †P < 0.05 and ††P < 0.01 versus week 4 by repeated measures ANOVA followed by Bonferroni’s post hoc analysis. AD, atopic dermatitis; N.S., not significant by paired t-test; pCer, pseudoceramide; SC, stratum corneum; TEWL, transepidermal water loss.
acids, o-hydroxy fatty acids, and sphingosines (Cer[EOS]); ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and 6-hydroxysphingosines (Cer[EOH]); and ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and phytosphingosines (Cer[EOP]) significantly decreased, which indicates that the ceramide profile of AD skin acquires a feature characteristic of healthy skin (Ishikawa et al., 2010).

**Changes in ceramide carbon numbers before and after 4 weeks of treatment with the pCer lotion**

Comparisons of ceramide carbon numbers in each ceramide species before and after 4 weeks of treatment with the pCer lotion revealed that the average alkyl chain lengths of Cer[NS] and Cer[ADS] significantly increased (Figure 4). In contrast, the average alkyl chain lengths of Cer[NDS], Cer[AP], Cer[AS], Cer[AH], Cer[EOS], Cer[EOH], and Cer[EOP] became significantly shorter, whereas those of ceramides containing nonhydroxy fatty acids and dihydrosphingosines (Cer[NDS]) and Cer[NH] remained unchanged. The distribution of total carbon numbers of endogenous ceramides in the SC of nonlesional AD skin before and after treatment with the pCer lotion are mainly attributable to the decreased levels of ceramide species with shorter alkyl chain lengths and the increased levels of ceramide species with longer alkyl chain lengths in the Cer[NS] and Cer[ADS] classes, respectively (Supplementary Figure S3). In contrast, there were no distinct changes in the distribution of total carbon numbers of endogenous ceramide species with shorter or longer alkyl chain lengths in the other ceramide species (Cer[NDS], Cer[NH], Cer[AP], Cer[AS], Cer[AH], Cer[EOS], Cer[EOH], Cer[EOH], and Cer[EOP]). However, there were increased or decreased levels of ceramide species without distinct distribution changes of alkyl chain lengths in the Cer[NH], Cer[AP], Cer[AS], and Cer[AP] classes and the Cer[EOS], Cer[EOH] and Cer[EOP] classes, respectively. Thus, a comparison between average alkyl chain length and its distribution indicates that the increased average alkyl chain lengths in Cer[NS] and Cer[ADS] after treatment with the pCer lotion are mainly attributable to the decreased levels of ceramide species with shorter alkyl chain lengths and the increased levels of ceramide species with longer alkyl chain lengths, respectively.

**Relationships between levels of ceramide classes and penetrated pCer and skin condition-associated factors**

Although there were no significant correlations between all endogenous ceramide species and skin condition-associated factors revealed that there is a
The changes in the average of total carbon numbers of endogenous ceramide species in the SC of nonlesional AD skin on the forearm before and after the 4 weeks of treatment with the pCer lotion. n = 38, data represent individual values and mean ± SD. P-values were analyzed by paired t-test. AD, atopic dermatitis; Cer[ADS], ceramides containing a-hydroxy fatty acids and dihydrosphingosines; Cer[AH], ceramides containing a-hydroxy fatty acids and 6-hydroxysphingosines; Cer[AP], ceramides containing a-hydroxy fatty acids and phytosphingosines; Cer[AS], ceramides containing a-hydroxy fatty acids and sphingosines; Cer[EAS], ceramides containing ester-linked fatty acids, a-hydroxy fatty acids, and dihydrosphingosines; Cer[EOH], ceramides containing ester-linked fatty acids, a-hydroxy fatty acids, and 6-hydroxysphingosines; Cer[EOP], ceramides containing ester-linked fatty acids, a-hydroxy fatty acids, and phytosphingosines; Cer[EOS], ceramides containing ester-linked fatty acids, a-hydroxy fatty acids, and sphingosines; Cer[NDS], ceramides containing nonhydroxy fatty acids and dihydrosphingosines; Cer[NH], ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines; Cer[NP], ceramides containing nonhydroxy fatty acids and phytosphingosines; Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; N.S., not significant; SC, stratum corneum; pCer, pseudoceramide.

significant correlation (r = 0.447, P = 0.005) with the SC water content but not with TEWL (Supplementary Table S1) (Figure 5). That suggests that pCer that penetrated into the SC contributes mainly to the increased water-holding function in the SC.

Relationships between levels of pCer that penetrated into the SC and endogenous ceramide profiles

We next determined the relationships between the levels of pCer that penetrated into the SC and endogenous ceramide profiles. Comparison of levels of pCer that penetrated into the SC with endogenous ceramide species (expressed as weight %) indicated significant correlations only between the levels of Cer[NS] (r = -0.325, P = 0.047) and average carbon chain of Cer[NS] (r = 0.393, P = 0.015) and the penetrated levels of pCer (Supplementary Table S2) (Figure 5).

DISCUSSION

In this clinical study, daily continued use of the pCer-containing lotion on mild to severe AD skin improved the clinical symptoms of both lesional and nonlesional skin of patients with AD. These benefits increased progressively during the four weeks of treatment and continued even through the regression phase (7 days). Improvements of the skin symptoms were confirmed by the investigator’s clinical scoring and by instrumental measures for barrier (TEWL) and water-holding functions (conductance). Significant benefits from the pCer lotion were noted specifically in the clinical scoring of erythema. Taken together, this study demonstrates the value of the pCer lotion to reduce the clinical symptoms associated with mild to severe AD as well as improving skin barrier and water-holding functions.

To elucidate the cutaneous mechanisms involved in the improvements of skin symptoms as well as the SC barrier and water-holding functions by treatment with the pCer lotion, we found that, whereas the decreased levels of endogenous ceramides in the SC of treated nonlesional AD skin remained unchanged after 4 weeks of treatment, the ceramide profiles of endogenous ceramides, even though present at still decreasing levels, were significantly altered in terms of their species and carbon chain number. In contrast, the pCer applied was additionally accumulated at a similar average level to existing endogenous ceramides in the treated nonlesional SC of AD skin. These findings suggest the following possibilities as the biological mechanisms involved in the improvement of skin symptoms and SC functions:

1. The changes in ceramide profiles of endogenous ceramides in the SC of AD skin are in part responsible for the improvements.
Ceramides in human SC have been assigned to 12 distinct groups based upon their fatty acid and sphingoid base structures (Masukawa et al., 2008), as shown in Supplementary Figure S4. In the skin of patients with AD, Ishikawa et al. (2010) used normal-phase liquid chromatography–electrospray ionization mass spectrometry to demonstrate that the levels of endogenous ceramides are significantly downregulated in lesional SC, which is consistent with previous studies using other analytical methods (Arikawa et al., 2002; Choi and Maibach, 2005; Di Nardo et al., 1998; Imokawa et al., 1991a; Ishibashi et al., 2003; Ishikawa et al., 2010; Jungersted et al., 2010; Kim et al., 2017; Okamoto et al., 2003; Sugiuara et al., 2014). In contrast, whereas the levels of Cer[NH], Cer[NP], Cer[ADS], Cer[AH], and Cer[AP] significantly increased in the SC of AD skin after 4 weeks of treatment with the pCer lotion, levels of Cer[NS], Cer[AS], Cer[EOS], Cer[EOP], and Cer[EOH] significantly decreased. Although the average alkyl chain lengths of Cer[NS] and Cer[ADS] significantly increased, the average alkyl chain lengths of Cer[NP], Cer[AS], Cer[AH], and Cer[AP] tended to decrease, and those of Cer[NDS] and Cer[NH] remained unchanged. Therefore, based upon the report by Ishikawa et al. (2010), it is likely that the increased levels in the Cer[NS], Cer[NP] classes as well as the increase of average carbon number in Cer[NS] classes observed in this study could be considered as a possible shift from an AD phenotype to a healthy phenotype of ceramide profiles, acquiring a

(2) The penetrated and accumulated pCer plays an essential role in ameliorating the SC barrier and water-holding functions, in turn leading to the improved skin symptoms.

In this study, the improvement of skin symptoms and barrier and water-holding functions by 4 weeks of treatment with the pCer lotion was accompanied by distinct changes in ceramide profiles, including carbon chains. Whereas the levels of Cer[NH], Cer[NP], Cer[ADS], Cer[AH], and Cer[AP] significantly increased in the SC of AD skin after 4 weeks of treatment with the pCer lotion, levels of Cer[NS], Cer[AS], Cer[EOS], Cer[EOP], and Cer[EOH] significantly decreased. Although the average alkyl chain lengths of Cer[NS] and Cer[ADS] significantly increased, the average alkyl chain lengths of Cer[NP], Cer[AS], Cer[AH], and Cer[AP] tended to decrease, and those of Cer[NDS] and Cer[NH] remained unchanged. Therefore, based upon the report by Ishikawa et al. (2010), it is likely that the increased levels in the Cer[NH] and Cer[NP] classes as well as the increase of average carbon number in Cer[NS] classes observed in this study could be considered as a possible shift from an AD phenotype to a healthy phenotype of ceramide profiles, acquiring a

![Figure 5. Relationships between pCer level penetrated into the SC and skin condition-associated factors and endogenous Cer[NS] level after 4 weeks of treatment with the pCer lotion.](image-url)

(a) pCer level versus skin conductance. n = 38, data represent individual values and linear regression. Correlation coefficient $r = 0.447$ and $P = 0.005$ were analyzed by Pearson’s correlation coefficient. (b) pCer level versus TEWL. n = 38, data represent individual values and linear regression. Correlation coefficient $r = 0.101$ and $P = 0.545$ were analyzed by Pearson’s correlation coefficient. (c) pCer level versus class ratio (weight %) of Cer[NS], n = 38, data represent individual values and linear regression. Correlation coefficient $r = -0.325$ and $P = 0.047$ were analyzed by Pearson’s correlation coefficient. Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; pCer, pseudoceramide; SC, stratum corneum; TEWL, transepidermal water loss.

(b) The penetrated and accumulated pCer plays an essential role in ameliorating the SC barrier and water-holding functions, in turn leading to the improved skin symptoms.
As for the association of structural ceramide profile changes with distinct improvements of the SC barrier and water-holding functions by treatment with the pCer lotion, in this study, there was no significant correlation between any ceramide class and skin condition—associated values and barrier and water-holding functions. It is likely that the failure of endogenous ceramide levels to increase by treatment with the pCer lotion results in no substantial contribution to the improvement of SC functions. In contrast, the relationship between the penetrated levels of pCer and skin condition—associated factors revealed that there is a significant correlation of the penetrated pCer levels with the SC water content but not with the barrier function (TEWL). Based on the much lower impaired levels of barrier function (TEWL) in the skin of patients with AD enrolled in this study compared with those in the study by Ishikawa et al. (2010), the stated findings strongly suggest that the penetrated and accumulated pCer in the SC contributes mainly to the increased water-holding function in the SC, resulting in the amelioration of inflammatory conditions, which is associated with the improvement of skin symptoms. The penetrated levels of pCer are only significantly correlated with the decreased levels of the Cer[NS] class as well as the increased levels of average carbon chain lengths of the Cer[NS] class among all Cer classes. Thus, the levels and the average carbon numbers of Cer[NS], which are characteristically increased or shortened as a result of the increased levels of shorter carbon number—bearing ceramides, respectively, in the SC of AD skin (Ishikawa et al., 2010) tended to decrease and become longer with the decreased levels of shorter carbon number—bearing ceramides, respectively, by treatment with the pCer lotion in a proportional fashion with the penetrated levels of the pCer. These results strongly suggest that the penetration and accumulation of pCer distinctly contributes to the preferable shift of the ceramide profile from an AD phenotype to a healthy phenotype, accompanied by the amelioration of itchy or dry skin mainly because of the improvement of downregulated levels of water content in the SC of AD skin.

In the mechanistic linkage between the altered ceramide profiles, especially for the reduced levels of Cer[NS] with long-chain fatty acids and causative inflammatory factors such as cytokines, Tawada et al. (2014) speculated that a potent proinflammatory T helper type 1—type cytokine, IFN-γ, whose mRNA and protein levels are distinctly upregulated in lesional AD skin during the cutaneous inflammatory process (Bieber, 2010; Grewe et al., 1998, 1994), is mainly associated with the decreased levels of ceramides with long-chain fatty acids resulting from the downregulated expression levels of ELOVL1 (Ohno et al., 2010) and ceramide synthase 3 (Mizutani et al., 2008). In this study, because inflammatory reactions were remarkably diminished by the topical treatment with the pCer lotion, it is likely that the levels of IFN-γ may be downregulated in the pCer-treated AD skin, thus supporting the possibility that treatment with pCer ameliorates the dermatitis by repairing the impaired barrier and water-holding functions. That may result in the downregulation of IFN-γ expression, leading to the recovery of levels of Cer[NS] with long-chain fatty acids.

In conclusion, an intriguing point for the observed clinical improvements by the pCer lotion is that these clinical efficacies can be achieved without any recovery of the decreased levels of endogenous ceramides but with compensated pCer levels similar to existing endogenous ceramides, despite the fact that the ceramide profiles shifted from an AD phenotype to a healthy phenotype in the pCer lotion—treated SC of AD skin. This suggests that ceramide levels in the SC are more essential to maintaining the barrier and water-holding functions than are the differential ceramide profiles. This hypothesis is supported by the fact that altered ceramide profiles in AD skin are not atopy-specific but are inflammation-specific and can be impaired by upregulating the barrier and water-holding functions. Thus, the observed clinical efficacy using the pCer lotion provides a deep insight into the pathogenesis of AD as a ceramide-deficient disease.

MATERIALS AND METHODS

Materials
The pCer test lotion was an oil-in-water—type lotion that contains 3% of Cetyl-PG hydroxyethyl palmitamide as a pseudoceramide (see Supplementary Materials for more information about the pCer and lotion formula).

Study design overview
This study was conducted in an open-label, single-center method. Subjects with mild to severe AD were enrolled in this 6-week study. Each subject completed a washout of all topical medications and lotions for 1 week, then applied the pCer test lotion (whose
formulation is shown in Supplementary Table S3) twice daily (morning and evening) for 4 weeks. At the completion of the four weeks of treatment, subjects discontinued the use of the pCer test lotion for a 1-week regression period. During the study, subjects did not use any medications on their skin and all skin care products remained unchanged and no new products were added. All subjects underwent skin assessments at the onset of the treatment phase (baseline, i.e., week 0); 24 hours after the first application of the test lotion; and after 1, 2, and 4 weeks of treatment. Investigator assessments were performed at baseline and at the completion of 1, 2, and 4 weeks of treatment. Assessments of TEWL were performed at baseline and at weeks 1, 2, and 4. Corneometry was performed at baseline, 24 hours, and weeks 1, 2 and 4. Tape-stripping for ceramide sampling was performed at baseline and at week 4. A subset of 10 subjects participated in surface imaging at baseline, 24 hours, week 2, and week 4, as well as a methyl nicotinate insult for assessing barrier function at baseline and at week 4. All assessments were made at least 12 hours following test sample application. During the regression phase, subjects returned to the test facility for evaluation at day 3 and day 7, and all subjects participated in TEWL and corneometry assessments on both days. A subset of 10 subjects also underwent surface imaging and barrier function testing at day 7.

Study population
This study enrolled 38 subjects. The demographics of the study group were 36 females and 2 males, ranging from 29 to 71 years old, and included 21 African Americans and 17 Caucasians. AD severity, according to the diagnostic criteria of Hanifin and Rajka (1980), were 14 mild, 15 moderate, and 9 severe.

Study approval
This test was registered under the number UMIN000037554. Ethics approval was obtained on the basis of the study protocol (study number: DCS-79-09) by the institutional review board of Concordia Clinical Research (Cedar Knolls, NJ, USA, IRB number: 175V). Written informed consent was obtained from each subject before performing any study procedures.

Clinical assessment
A dermatologist (ZD) performed all clinical assessments. The evaluation parameters consisted of stinging, burning, itching (sensory attributes assessed by querying the subject), erythema, desquamation, lichenification and excoriation. The following rating scale was used for each evaluation: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe.

Skin conductance and TEWL measurements
Skin conductance and TEWL values were measured using a DermaLab Moisture Meter with a pin probe and a DermaLab TEWL module, respectively. These measurements were taken at both lesional and nonlesional skin sites according to the methods previously reported (Draelos and Raymond, 2018).

Statistical analyses
Statistical analyses were performed using SPSS for Windows, version 25 (SPSS Inc., Chicago, IL). For the changes over time, Wilcoxon signed-rank test, repeated measures ANOVA followed by Bonferroni’s post hoc analysis, or paired t-test was used. For correlation studies, Pearson’s correlation coefficient was used. All data are expressed as mean ± SD, and a P-value of 0.05 or less was considered statistically significant. Other details of the Materials and the Methods used (sources of materials, barrier function test, and ceramide assessment method) can be found in Supplementary Materials and Methods.

Data availability statement
No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST
KI, AT, and KB have been employed by Kao Corporation. ZD received an educational grant from Kao to conduct the research detailed in this manuscript. GI states no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: KI, AT, KB; Formal Analysis: GI, KI; Investigation: KI, AT, KB, ZD; Writing - Original Draft Preparation: GI, KI

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org and at https://doi.org/10.1016/j.jid.2020.01.014.

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SUPPLEMENTARY MATERIALS AND METHODS

Materials
Cetyl-PEG hydroxyethyl palmitamide (Supplementary Figure S5) (Imokawa, 2004, 2001, 1999, 1994; Imokawa et al., 1989a, 1989b; Matsuki et al., 2004; Takagi et al., 2005), developed by Kao Corporation (Tokyo, Japan), was used as the synthetic pseudoceramide (pCer). An oil-in-water—type lotion containing 3% pCer and 0.9% eucalyptus extract that is known to increase ceramide content in the stratum corneum (Ishikawa et al., 2012) was used as the test lotion for this study. The formula of the pCer test lotion is shown in Supplementary Table S3.

Barrier function test
The methyl nicotinate insult method was used to evaluate the barrier function of the volar forearm skin. A 1-cm diameter Whatman filter paper #1 disc was placed on the test site, and the filter paper disc was removed from the skin (blotted dry if necessary), and the erythema index was measured using a Cortex Technology DSM II Color meter. Transient sensations by subject assessment were evaluated immediately and at 5, 10, and 15 minutes after removal of the disc.

Ceramide assessment method
The level and total carbon number of endogenous ceramides and pCer in the stratum corneum were determined by HPLC—mass spectrometry analysis of tape strips taken from the volar surface of the forearms of all subjects. At the specified sampling intervals (baseline and week 4), each subject had a nonlesional volar surface of the forearm, approximately 2.5 cm × 2.5 cm, stripped 10 times with a polyphenylene sulfide film tape (Nichiban, Tokyo, Japan). All tape strips were divided in half so that one portion was designated for analysis of total soluble protein and the other half was designated for the determination of ceramides and pCer contents. For each subject, tapes from strippings 2 through 10 were combined for extraction and analysis. Ceramides were extracted from half of the tape-stripped specimens according to a previously described method (Sugiura et al., 2014). The concentration of soluble proteins was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with a calibration curve established using BSA as a standard (Smith et al., 1985). Analysis of the ceramides and pCer in the tape strips was performed according to normal-phase liquid chromatography—electrospray ionization mass spectrometry, as described previously (Masukawa et al., 2009, 2008), which can separate ceramides in the extracted lipid mixture with normal-phase gradient liquid chromatography, and ceramides were detected using their m/z of molecular-related ions in the selected ion monitoring mass spectrometry, as shown in part by Supplementary Figure S6. An Agilent 1100 Series LC/MSD single-quadrupole system equipped with an electrospray ionization source (Agilent Technologies, Santa Clara, CA) and an Inertsil SIL 100A-3, 1.5 mm inner diameter × 150 mm column (GL Science, Tokyo, Japan) were used in the analysis. The levels of each ceramide were quantified by using N-stearoyl-D-erythro-sphingosine (C18 Ceramide [d17:1/18:0] Avanti Polar Lipids, Alabaster, AL) as an internal standard, according to specific equations for quantification of all ceramide species that were established by using synthetic natural ceramide standards (Masukawa et al., 2009).

SUPPLEMENTARY RESULTS

Change in the permeability barrier
Evaluation of the permeability barrier by the methyl nicotinate test indicated that in the nontreated atopic dermatitis skin (at baseline), erythema induced by methyl nicotinate was significantly accentuated at 10 and 15 minutes after application (Supplementary Figure S7). In the pCer lotion—treated nonlesional atopic dermatitis skin at week 4 and in the regression phase, erythema did not significantly appear at 5, 10, and 15 minutes after application. These findings suggest that the penetration (outside-in) barrier in the pCer-treated skin was markedly improved compared with the baseline.

SUPPLEMENTARY REFERENCES

Supplementary Figure S1. Assessments of skin symptoms during 4 weeks of treatment with the pCer lotion. n = 38, data represent the percentage of severity score for each skin symptom. pCer, pseudoceramide.
Supplementary Figure S2. Changes in the class ratio (weight %) of endogenous ceramide species in the SC of nonlesional AD skin on the forearm. Before and after 4 weeks of treatment with the pCer lotion. n = 38, data represent individual values and mean ± SD. P-values were analyzed by paired t-test. AD, atopic dermatitis; Cer[ADS], ceramides containing α-hydroxy fatty acids and dihydrosphingosines; Cer[AH], ceramides containing α-hydroxy fatty acids and 6-hydroxysphingosines; Cer[AP], ceramides containing α-hydroxy fatty acids and phytosphingosines; Cer[AS], ceramides containing α-hydroxy fatty acids and sphingosines; Cer[AEOH], ceramides containing ester-linked fatty acids, α-hydroxy fatty acids, and 6-hydroxysphingosines; Cer[EOP], ceramides containing ester-linked fatty acids, α-hydroxy fatty acids, and phytosphingosines; Cer[NDS], ceramides containing nonhydroxy fatty acids and dihydrosphingosines; Cer[NH], ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines; Cer[NP], ceramides containing nonhydroxy fatty acids and phytosphingosines; Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; N.S., not significant; SC, stratum corneum.
Supplementary Figure S3. Changes in the total carbon number distributions of endogenous ceramide species in the SC of nonlesional AD skin on the forearm. Before and after treatment with the pCer lotion for 4 weeks, n = 38, data represent mean ± SD and moving averages. (a) Cer[NDS]. (b) Cer[NS]. (c) Cer[NH]. (d) Cer[NP]. (e) Cer[ADS]. (f) Cer[AS]. (g) Cer[AH]. (h) Cer[AP]. (i) Cer[EOS]. (j) Cer[EOP]. (k) Cer[EOH]. (l) Cer[NDS]. (m) Cer[AP]. (n) Cer[ADS]. AD, atopic dermatitis; Cer[ADS], ceramides containing a-hydroxy fatty acids and dihydrosphingosines; Cer[AH], ceramides containing a-hydroxy fatty acids and 6-hydroxysphingosines; Cer[AP], ceramides containing a-hydroxy fatty acids and phytosphingosines; Cer[AS], ceramides containing a-hydroxy fatty acids and sphingosines; Cer[EOH], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and 6-hydroxysphingosines; Cer[EOP], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and phytosphingosines; Cer[EOS], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and sphingosines; Cer[NDS], ceramides containing nonhydroxy fatty acids and dihydrosphingosines; Cer[NH], ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines; Cer[NP], ceramides containing nonhydroxy fatty acids and phytosphingosines; Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; pCer, pseudoceramide; SC, stratum corneum.
Supplementary Figure S4. Chemical structures of endogenous ceramide species in the SC of human skin.
Ceramides in human SC can be divided into 11 groups according to their fatty acid and sphingoid base structures. CER[NDS] contains nonhydroxy fatty acids [N] and dihydro sphingosines [DS]; CER[NS] contains [N] and sphingosines [S]; CER[NH] contains [N] and 6-hydroxysphingosines [H]; CER[NP] contains [N] and phytosphingosines [P]; CER[ADS] contains α-hydroxy fatty acids [A] and [DS]; CER[AS] contains [A] and [S]; CER[AH] contains [A] and [H]; CER[AP] contains [A] and [P]; CER[EOS] contains ester-linked fatty acids and α-hydroxy fatty acids [EO] and [S]; CER[EOP] contains [EO] and [H]; and CER[EOP] contains [EO] and [P]. These classes can be further subdivided into many species based on their chain length. To date, more than 350 species of ceramides have been identified in human SC using NPLC-ESI-MS. NPLC-ESI-MS, normal-phase liquid chromatography—electrospray ionization mass spectrometry; SC, stratum corneum.

Supplementary Figure S5. Chemical structure of natural ceramide and synthetic pCer. (a) Natural ceramide: Cer[NS]. (b) Product name of pCer: SOFCARE CERAMIDE SL-E (Kao Corporation), INCI Name: Cetyl-PEG hydroxethyl palmitamide, Molecular weight: 598 (C37H75O4N), Melting point: 69.0°C, Purity >97%. Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; pCer, pseudoceramide.
Supplementary Figure S6. Multi-SIM chromatograms of ceramide species in the SC using NPLC-ESI-MS. (a) At the baseline and (b) after 4 weeks of use of the pCer test lotion. Internal standard: N-stearoyl-D-erythro-sphingosine (Avanti Polar Lipids, Alabaster, AL). NPLC-ESI-MS, normal-phase liquid chromatography—electrospray ionization mass spectrometry; SC, stratum corneum; SIM, selected ion monitoring.

Supplementary Figure S7. Change of the skin barrier function evaluated by the erythema index. Function was evaluated by color meter after using the pCer lotion for 4 weeks and after the 7 days regression phase, following application of methyl nicotinate on the nonlesional AD skin on the volar forearm. n = 9, data represent mean ± SD. **P < 0.01 versus baseline analyzed by repeated measures ANOVA and post hoc Bonferroni correction. AD, atopic dermatitis; pCer, pseudoceramide.
Supplementary Table S1. Relationships between Endogenous Ceramide Species and Penetrated Levels of pCer and Skin Condition—Associated Factors

<table>
<thead>
<tr>
<th>pCer and Endogenous Ceramide Species</th>
<th>Conductance (µS)</th>
<th>TEWL (gm/m²/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P-value</td>
</tr>
<tr>
<td>pCer</td>
<td>0.447</td>
<td>0.005¹</td>
</tr>
<tr>
<td>Total ceramides</td>
<td>−0.224</td>
<td>0.177</td>
</tr>
<tr>
<td>Cer[NDS]</td>
<td>0.213</td>
<td>0.199</td>
</tr>
<tr>
<td>Cer[NS]</td>
<td>−0.198</td>
<td>0.232</td>
</tr>
<tr>
<td>Cer[NH]</td>
<td>−0.194</td>
<td>0.243</td>
</tr>
<tr>
<td>Cer[NP]</td>
<td>−0.135</td>
<td>0.419</td>
</tr>
<tr>
<td>Cer[ADS]</td>
<td>−0.178</td>
<td>0.286</td>
</tr>
<tr>
<td>Cer[AS]</td>
<td>−0.224</td>
<td>0.177</td>
</tr>
<tr>
<td>Cer[AH]</td>
<td>−0.207</td>
<td>0.213</td>
</tr>
<tr>
<td>Cer[AP]</td>
<td>−0.244</td>
<td>0.141</td>
</tr>
<tr>
<td>Cer[EOS]</td>
<td>−0.198</td>
<td>0.233</td>
</tr>
<tr>
<td>Cer[EOH]</td>
<td>−0.204</td>
<td>0.219</td>
</tr>
<tr>
<td>Cer[EOP]</td>
<td>−0.184</td>
<td>0.270</td>
</tr>
</tbody>
</table>

Abbreviations: AD, atopic dermatitis; Cer[ADS], ceramides containing a-hydroxy fatty acids and dihydrosphingosines; Cer[AH], ceramides containing a-hydroxy fatty acids and 6-hydroxysphingosines; Cer[AP], ceramides containing a-hydroxy fatty acids and phytosphingosines; Cer[AS], ceramides containing a-hydroxy fatty acids and sphingosines; Cer[EOH], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and 6-hydroxysphingosines; Cer[EOP], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and phytosphingosines; Cer[EOS], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and sphingosines; Cer[NDS], ceramides containing nonhydroxy fatty acids and dihydrosphingosines; Cer[NH], ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines; Cer[NP], ceramides containing nonhydroxy fatty acids and phytosphingosines; Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; pCer, pseudoceramide; SC, stratum corneum; TEWL, transepidermal water loss.

Ceramide levels were measured in ng/µg protein in the SC of nonlesional AD skin on the forearm after 4 weeks of use of the pCer lotion. n = 38, correlation coefficients and P-values were analyzed by Pearson’s correlation coefficient.

¹P < 0.01.

Supplementary Table S2. Relationships between pCer Levels and Class Ratio of Ceramide Species and Average of Total Carbon Number of Endogenous Ceramides

<table>
<thead>
<tr>
<th>Endogenous Ceramide Species</th>
<th>Class ratio of Ceramides (weight %)</th>
<th>Average of total carbon number of Ceramides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Cer[NDS]</td>
<td>−0.185</td>
<td>0.265</td>
</tr>
<tr>
<td>Cer[NS]</td>
<td>−0.325</td>
<td>0.047¹</td>
</tr>
<tr>
<td>Cer[NH]</td>
<td>−0.090</td>
<td>0.590</td>
</tr>
<tr>
<td>Cer[NP]</td>
<td>0.126</td>
<td>0.450</td>
</tr>
<tr>
<td>Cer[ADS]</td>
<td>0.027</td>
<td>0.871</td>
</tr>
<tr>
<td>Cer[AS]</td>
<td>−0.117</td>
<td>0.484</td>
</tr>
<tr>
<td>Cer[AH]</td>
<td>0.021</td>
<td>0.902</td>
</tr>
<tr>
<td>Cer[AP]</td>
<td>0.105</td>
<td>0.529</td>
</tr>
<tr>
<td>Cer[EOS]</td>
<td>0.091</td>
<td>0.586</td>
</tr>
<tr>
<td>Cer[EOH]</td>
<td>0.085</td>
<td>0.613</td>
</tr>
<tr>
<td>Cer[EOP]</td>
<td>0.193</td>
<td>0.245</td>
</tr>
</tbody>
</table>

Abbreviations: AD, atopic dermatitis; Cer[ADS], ceramides containing a-hydroxy fatty acids and dihydrosphingosines; Cer[AH], ceramides containing a-hydroxy fatty acids and 6-hydroxysphingosines; Cer[AP], ceramides containing a-hydroxy fatty acids and phytosphingosines; Cer[AS], ceramides containing a-hydroxy fatty acids and sphingosines; Cer[EOH], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and 6-hydroxysphingosines; Cer[EOP], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and phytosphingosines; Cer[EOS], ceramides containing ester-linked fatty acids, o-hydroxy fatty acids, and sphingosines; Cer[NDS], ceramides containing nonhydroxy fatty acids and dihydrosphingosines; Cer[NH], ceramides containing nonhydroxy fatty acids and 6-hydroxysphingosines; Cer[NP], ceramides containing nonhydroxy fatty acids and phytosphingosines; Cer[NS], ceramides containing nonhydroxy fatty acids and sphingosines; pCer, pseudoceramide; SC, stratum corneum.

pCer levels were measured in ng/µg protein in the SC of nonlesional AD skin on the forearm after 4 weeks of use of the pCer lotion. Class ratio was measured in weight %. n = 38, correlation coefficients and P-values were analyzed by Pearson’s correlation coefficient.

¹P < 0.05.
### Supplementary Table S3. pCer Test Lotion Formula

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase A</td>
<td>Cetyl-PG Hydroxyethyl Palmitamide (pCer); 3.0%</td>
</tr>
<tr>
<td></td>
<td>Bis-Methoxypropylamido Isodocosane</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
</tr>
<tr>
<td></td>
<td>Stearyl Alcohol / Cetyl Alcohol</td>
</tr>
<tr>
<td></td>
<td>Isopropyl Palmitate</td>
</tr>
<tr>
<td></td>
<td>Polyglyceryl-2 Diisostearate</td>
</tr>
<tr>
<td></td>
<td>Sorbitan Stearate</td>
</tr>
<tr>
<td></td>
<td>Polyoxyethylene (20) Sorbitan Monostearate</td>
</tr>
<tr>
<td>Phase B</td>
<td>Allantoin</td>
</tr>
<tr>
<td></td>
<td>Glycerin</td>
</tr>
<tr>
<td></td>
<td>Sodium Methyl Stearoyl Taurate</td>
</tr>
<tr>
<td></td>
<td>Citric Acid</td>
</tr>
<tr>
<td></td>
<td>Paraben</td>
</tr>
<tr>
<td></td>
<td>Deionized Water</td>
</tr>
<tr>
<td>Phase C</td>
<td>Eucalyptus Globulus Leaf Extract (BG/Water)</td>
</tr>
<tr>
<td></td>
<td>0.9% Dimethylpolysiloxane</td>
</tr>
</tbody>
</table>

Abbreviations: O/W, oil-in-water; pCer, pseudoceramide.

Preparation Method: O/W lotion was prepared by mixing phase A and phase B at 80–85°C, adding phase C, and cooling to room temperature.

pH of formulation (by 10-fold dilution): 4.5–5.0.