Decreased Level of Ceramides in Stratum Corneum of Atopic Dermatitis: An Etiologic Factor in Atopic Dry Skin?

Genji Imokawa, Akihito Abe, Kumi Jin, Yuko Higaki, Makoto Kawashima, and Akira Hidano
Tochigi Research Laboratories (GI,AA), Kao Corporation, Tochigi; and Department of Dermatology (KJ,YH,MK,AH), Tokyo Women's Medical College, Tokyo, Japan

Stratum corneum lipids are an important determinant for both water-retention function and permeability-barrier function in the stratum corneum. However, their major constituent, ceramides, have not been analyzed in detail in skin diseases such as atopic dermatitis that show defective water-retention and permeability-barrier function. In an attempt to assess the quantity of ceramides per unit mass of the stratum corneum in atopic dermatitis, stratum corneum sheet was removed from the forearm skin by stripping with cyanoacrylate resin and placed in hexane/ethanol extraction to yield stratum corneum lipids. The stratum corneum was dispersed by solubilization of cyanoacrylate resin with dimethylformamide, and after membrane filtration, the weight of the stratum corneum mass was measured. The ceramides were quantified by thin-layer chromatography and evaluated as $\mu g/mg$ stratum corneum. In the forearm skin of healthy individuals (n = 65), the total ceramide content significantly declined with increasing age. In atopic dermatitis (n = 32–35), there was a marked reduction in the amount of ceramides in the lesional forearm skin compared with those of healthy individuals of the same age. Interestingly, the non-lesional skin also exhibited a similar and significant decrease of ceramides. Among six ceramide fractions, ceramide 1 was most significantly reduced in both lesional and non-lesional skin. These findings suggest that an insufficiency of ceramides in the stratum corneum is an etiologic factor in atopic dry skin. *J Invest Dermatol 96:523–526, 1991*

Stratum corneum lipids serve as a water retainer [1] as well as permeability barrier [2–4] by forming a multi-lammellae structure in the stratum corneum. The major constituent of these lipids, ceramides, have been shown to be predominantly associated with both functions [5–7]. Quantitative evaluation of ceramide in the stratum corneum provides insight into etiologic involvement of ceramides in the dry skin of aged persons and in atopic dermatitis because these symptoms are accompanied by the diminished water permeability barrier and deficient water-holding properties, as revealed by a evaporimeter for transepidermal water loss [8,9] and by a capacitance conductance meter for skin surface water content [8,10], respectively. Especially, altered permeability to topical allergens or irritants may be associated with a fundamental role in the pathogenesis of atopic dermatitis. However, quantitative analysis of ceramides based on stratum corneum mass are lacking in such dry skin diseases. We have tried removing stratum corneum layers [11] to assess the quantity of ceramides per unit mass of the stratum corneum. The present studies are directed towards clarifying the role of ceramides in xerotic skin and atopic dry skin on the basis of their pathologic variation.

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Reprint requests to: Dr. Genji Imokawa, 1022-89 Himuro Utsunomiya, Tochigi 321-32, Japan.

Abbreviations:
AD: atopic dermatitis
DMF: dimethylformamide
SC: stratum corneum
TLC: thin-layer chromatogram

MATERIALS AND METHODS

Lipid Extraction Stratum corneum (SC) was obtained from the volar side of forearm skins of 65 healthy subjects (13 to 81 years old, mean age 42.5) and lesional or non-lesional skin of 35 patients with atopic dermatitis (0 to 30 years old, 23 men and 12 women). Diagnosis of atopic dermatitis was made according to Hanifin and Rajka [12]. Thirty-four healthy volunteers aged 0–30 years, 14 men and 20 women, were selected as controls for age-matched comparison with atopic individuals. All procedures for SC separation and treatment are summarized in Fig 1. Stratum corneum sheet was removed from the volar side of forearm skins by a single stripping with cyanoacrylate resin, two drops of which was placed on a glass slide and allowed to adhere for 1 min on the test area of the forearm (Fig 2). The stripped stratum corneum sheet, which was shown to possess at least five layers of stratum corneum as estimated from biopsied H&E sections (Fig 3), was incubated twice with hexane:ethanol (95/5) under ultrasonication (Sibata Scientific Technology Ltd. FU-10 type Ultrasonicator) for 20 min and subsequently rinsed twice with the same solvent each for 1 min. Hexane:ethanol (95/5) was selected as an appropriate solvent for extracting all lipids present in stripped stratum corneum under the ultrasonication, but not solubilizing cyanoacrylate resin. Efficiency of this extraction was confirmed by the experiments that 100 μg of each lipid (triolein, free cholesterol, cholesterol oleate, stearic acid, ceramide type III [Sigma], ceramide type V [Sigma] and cholesterol sulfate) was completely solubilized by 15 ml of hexane:ethanol

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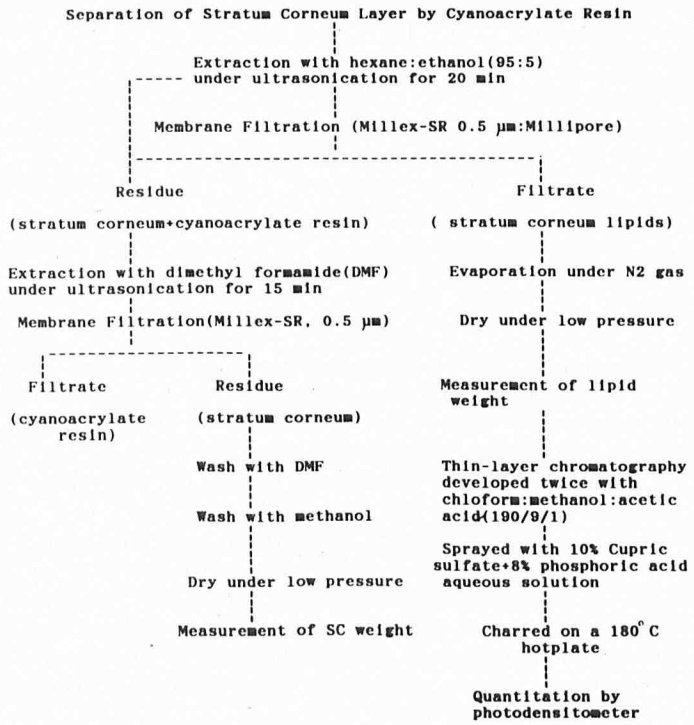

Figure 1. Method for quantitation of ceramides in human stratum corneum.

(95/5) with the help of 20-min ultrasonication. Furthermore, the values (μg total lipids/mg stratum corneum) that were obtained from two extractions with hexane : ethanol (95/5) under ultrasonification for 20 min, using stratum corneum samples stripped with cyanoacrylate resin, were substantially similar to those from extraction with chloroform : methanol (2/1) using the stratum corneum sheet that was taken from the forearm skin of the same individual by a surgical knife. Extracted stratum corneum lipids were filtered through a solvent-resistant Millipore filter (Millex-SR 0.5 μm), taken to dryness under nitrogen and subjected to measurement of lipid weight after final drying in a vacuum. The residue on the glass slide, which was composed of stratum corneum and cyanoacrylate resin, was treated with dimethyl formamide (DMF) under ultrasonication for 15 min to solubilize cyanoacrylate resin. This treatment

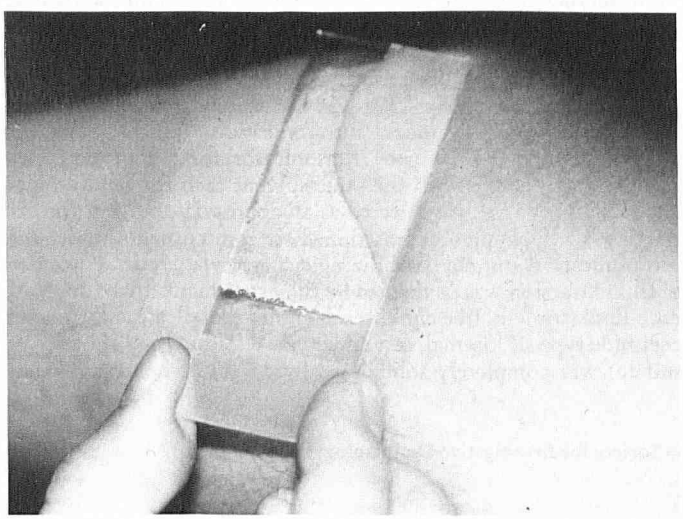

Figure 2. A photograph of stripping process by cyanoacrylate resin.

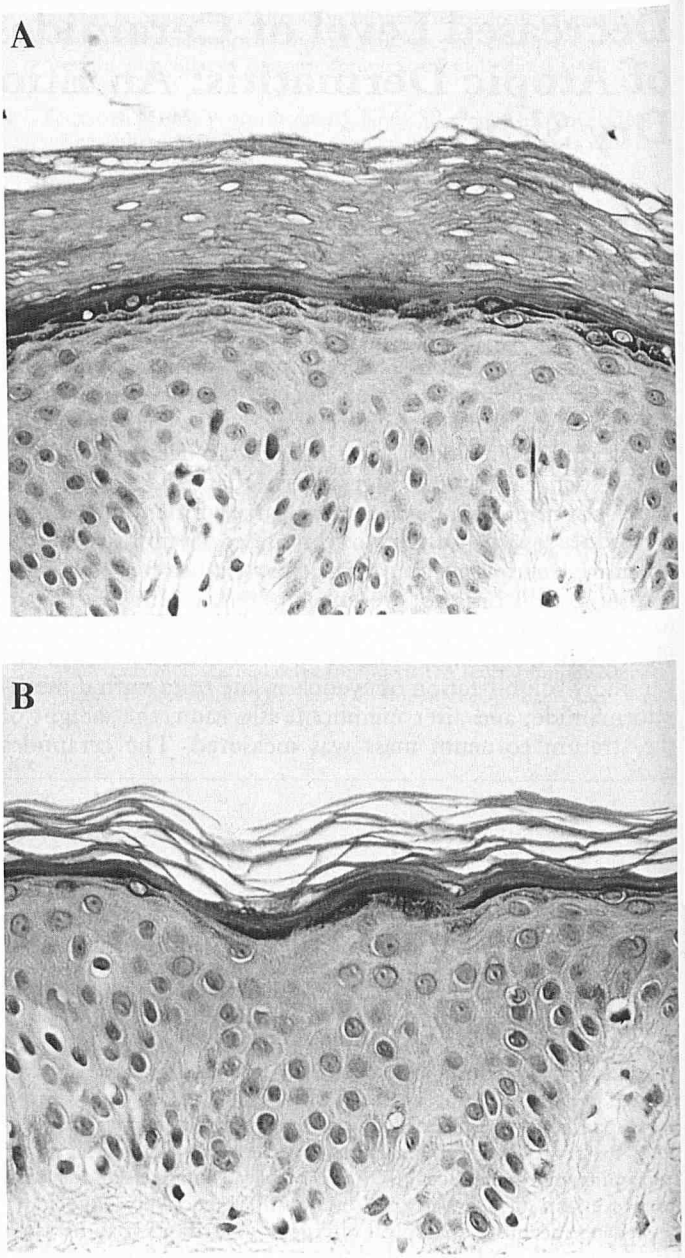

Figure 3. H&E section of forearm skin before and after stripping with cyanoacrylate resin, magnification ×400.

caused the stratum corneum to be dispersed in DMF solution. Dispersed stratum corneum was separated from DMF solution through filtration with the same Millipore filter as described above, and the resulting residue on the filter was subjected to measurement of stratum corneum weight after several washes with DMF/methanol and final dryness in vacuum. Under the separation procedure employed, lipids were completely extracted from the stripped stratum corneum and did not contain non-lipid debris. Similarly, the stratum corneum was completely separated from cyanoacrylate resin by solubilization with DMF.

Thin-layer Chromatograms Thin-layer chromatograms (TLC) were developed twice with chloroform: methanol: acetic acid (190 : 9 : 1) to resolve ceramides, cholesterol, cholesterol ester, triglyceride, free fatty acid, and cholesterol sulfate. After solvent development, the chromatograms were air-dried, sprayed with 10% $CuSO_3$, 8% H_3PO_4 aqueous solution and charred on a 180°C hotplate. The representative chromatogram is shown in Fig 4. The

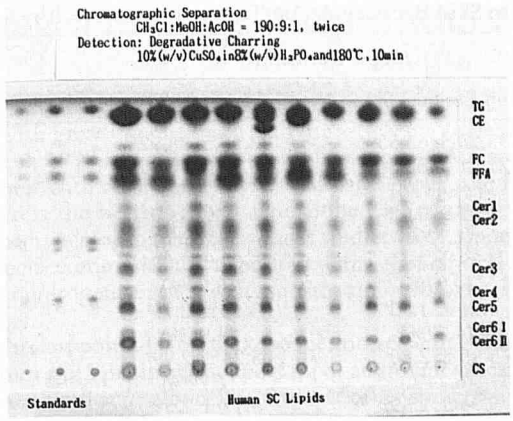

Figure 4. The representative thin-layer chromatogram of lipids extracted from stratum corneum that was stripped by cyanoacrylate resin.

charred lipids were quantitated by photodensitometry (Shimazu CS-9000) and their data were subjected to 2 Dimensional Image Analyzer (Shimazu). Ceramides were quantitated by determining μg of ceramides on a TLC chart from appropriate commercial standards and expressed as μg ceramide/mg stratum corneum weight. Cholesterol, cholesterol sulfate, cholesterol oleate, oleic acid, and triolein were used as lipid standard. Ceramide type III and ceramide type IV (Sigma Chemical Co.) were used as standards for ceramide 1 and 2, and ceramide 3, 4, 5, and 6, respectively. Reproducibility of this method was confirmed using triplicate samples from the same subjects and deviation of values (total lipids or ceramide μg/mg stratum corneum) was within 5% of the means.

Statistics The level of significance of the difference was calculated by the Student t test.

RESULTS

Figure 5 shows total ceramide content (μg) per mg stratum corneum as a function of age in healthy subjects (n = 65), demonstrating that the total ceramide content significantly declines with increasing age. Comparison of total ceramide content of forearm stratum corneum between atopic dermatitis and healthy subjects (Table I) shows that in atopic dermatitis, there is a marked reduction in the amount of total ceramides in both lesional and non-lesional forearm skins as compared with that of healthy individuals of the same age. Whereas the amounts of total lipids significantly decrease in atopic dermatitis, the ratio of total ceramides to total lipids slightly increases. Among six ceramide fractions, ceramide I is most significantly reduced in both lesional and non-lesional skin. The ratio of ceramide 1 to total lipids decreases slightly, although that of other ceramide fractions is not significantly altered in atopic individuals.

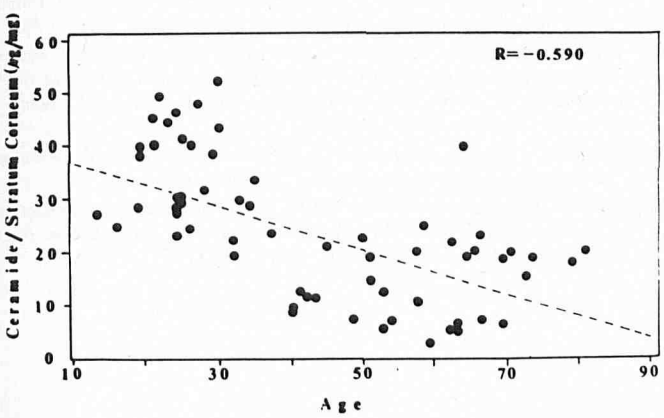

Figure 5. Total ceramide content (μg) per mg stratum corneum as a function of age in healthy subjects (n = 65), r = −0.59, p < 0.01.

DISCUSSION

The biochemical events underlying xerotic changes in atopic dermatitis skin are unknown. Available evidence [8–10,13] suggests that these symptoms are mainly associated with the diminished water-permeability barrier and deficient water-holding properties of atopic skin. Because both functions could be influenced by the presence of intercellular lipids in the stratum corneum, whose major lipid constituents are ceramides [5,6,14,15], we have evaluated quantitatively a possible alteration of ceramides in the stratum corneum of atopic dermatitis. For this assay, the solvent-extraction method of collecting lipids from the skin surface has been widely used [16], but it has proved difficult to know the exact variation of ceramides because of variations in extraction efficiency. Therefore, we have removed the stratum corneum sheet with specific glue and analyzed the amounts of ceramides per unit mass of stratum corneum.

The present study demonstrated that there is a distinct age dependency in the amounts of ceramides based on the mass of stratum corneum in healthy subjects. Because the forearm skin of aged persons (especially those over 70 years old) looks very xerotic, it is conceivable that the decrease in ceramide content is associated with dry appearance in xerotic skin. Although this is not in accord with a recent report describing no significant change in the percentage of various lipids in xerosis (17), our experiment also showed that total stratum corneum lipids/mg stratum corneum declines with age (data not shown), suggesting the total reduction of lipid synthesis in aged skin. The decreased ceramide content in aged skin is corroborated by a recent report [18] demonstrating that the activity of ceramide-producing enzyme, sphingomyelinase, in human epidermis decreases with aging.

In atopic dermatitis, not only lesional skin but also non-lesional skin exhibited a marked reduction in the quantity of ceramides. Of the ceramide fractions, ceramide 1, which is considered an essential component of permeability barrier function, is most greatly reduced. The ratio of total ceramides to total lipids increases somewhat in both lesional and non-lesional skin, which may be a reflection of the decrease in total lipids in atopic individuals. Whereas this approximately 50% reduction in total lipids in atopic individuals may also account for symptoms appearing in atopic individuals, our recent findings indicated that the presence of amphipathic lipids such as ceramides is a key factor in the formation of the lipid bilayers between adjacent stratum corneum cells, resulting in a water-holding function [19], because the stratum corneum is virtually devoid of phospholipids. Another key role of ceramides for water-holding properties and the barrier function of the stratum corneum was also found in in vivo recovery experiments [6,14,15,20] where, of the stratum corneum lipids, ceramides and acylceramides are highly effective in recovering water content and barrier function, respectively, in the lipid-depleted stratum corneum, even when topically and solely applied at equal percent concentrations of each lipid.

It is well established that the stratum corneum possesses approximately 30% water, which is mainly associated with its elasticity. Our recent differential scanning calorimetry study [21] demonstrated that the stratum corneum lipids possess almost the equivalent amount of bound water, which accounts for 10% of the water content within the stratum corneum. The remaining 20%, which is resistant to both solvent and water extraction, may be attributed to keratin components. Thus, the 10% difference that contributes to lipid-associated water plays a crucial role in keeping the skin supple and smooth. Because it is logical that the enhanced permeability to topical irritant or allergens is deeply involved in a fundamental factor in the pathogenesis of atopic dermatitis, the observed decrease by more than 30% in ceramides, the major constituent of the stratum corneum lipids, even in non-lesional skin of atopic individuals may indicate a possible etiologic involvement of ceramide quantity in aged and atopic dry skin. Because ceramides are produced during the keratinization process, and there is a report [22] of a disturbance of lamellar bodies in dry non-eczematous skin of patients with ato-

Table I. Comparison of Ceramide Content (μm/mg stratum corneum) in Forearm Skin Between Atopic Dermatitis and Healthy Subjects

	Healthy Age-Matched Controls	Atopic Dermatitis	
		Non-Lesional Skin	Lesional Skin
Total lipids	156.87 ± 95.50[a]	88.26 ± 63.53[c]	73.58 ± 38.92[c]
Total ceramide	31.41 ± 16.95 (20.00%)[b]	21.61 ± 8.75[c] (24.48%)	20.19 ± 10.39[c] (27.44%)
Ceramide 1	2.73 ± 2.13 (1.74%)	1.53 ± 0.93[c] (1.73%)	1.12 ± 0.80[c] (1.52%)
Ceramide 2	6.59 ± 3.99 (4.20%)	4.39 ± 2.43[c] (4.97%)	5.07 ± 2.98[c] (6.89%)
Ceramide 3	6.22 ± 3.69 (3.96%)	4.21 ± 1.93[c] (4.77%)	3.65 ± 2.79[c] (4.96%)
Ceramide 4 + 5	8.22 ± 4.44 (5.24%)	5.95 ± 2.80[c] (6.74%)	5.24 ± 2.99[c] (7.12%)
Ceramide 6	7.66 ± 4.18 (4.88%)	5.94 ± 2.59[d] (6.73%)	5.11 ± 2.59[c] (6.94%)
Age distribution (years)	n = 34	n = 35	n = 32
0–10	7	13	10
11–20	5	10	9
21–30	22	12	13
mean	18.7	14.4	17.4

[a] Mean ± SD.
[b] Percentage of total lipids.
[c] $p < 0.01$.
[d] $p < 0.05$ (compared with control).

pic dermatitis, it may be that the dry skin shares certain characteristics with the alteration of keratinization, which leads to the deficient synthesis of ceramides.

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