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Analysis of Beta-glucocerebrosidase and Ceramidase Activities in Atopic and Aged Dry Skin

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To elucidate the mechanisms that are involved in the decrease of ceramide levels in atopic dry skin and in aged skin, we examined both the activities of beta-glucocerebrosidase, which is a major enzyme in ceramide production, and of ceramidase, which is an essential enzyme in ceramide degradation, in the stratum corneum of atopic dry skin and aged skin. The specimens of the stratum corneum of forearm skin were obtained by tape-stripping from 61 healthy volunteers and 23 patients with atopic uninvolved skin. The beta-glucocerebrosidase activity in the stratum corneum extracts was estimated using fluorescent 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate. Ceramidase activity was determined using ^{14}C-palmitoylsphingosine as the substrate. Among the atopic skin samples, neither beta-glucocerebrosidase nor ceramidase activities were different from those of age-matched healthy controls. Nor was the beta-glucocerebrosidase activity deficient in the aged skin samples as compared to that seen in samples from the young, healthy group. In contrast, there was an age-related upregulation in ceramidase activity. The results indicate that the decrease of ceramides in atopic dry skin may not be accompanied by reduced synthesis or by enhanced degradation, each of which is primarily attributable to the above two enzymes, respectively. The pathogenesis of aged dry skin can be explained, at least partially, in terms of elevated ceramidase activity, which results in a disturbance of the lamellar structure of the stratum corneum lipids. ***Key words: atopic dermatitis; ceramide.***

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Recent studies have shown that stratum corneum lipids play an important role in both water-retention (1–3) and barrier function (4, 5) through the formation of a multi-lamellar structure between stratum corneum cells. Stratum corneum lipids are produced by keratinocytes during the keratinization process. Ceramides comprise more than 50% of the stratum corneum lipids and are major components of both functions (2, 5, 6). Quantitative analysis of ceramides in dry skin disorders is promising, since it may provide valuable information on the etiological involvement of stratum corneum lipids.

We previously evaluated ceramide levels per unit mass as measured in the stratum corneum of two dry skin conditions, atopic and aged dry skin (7). In those studies, we demonstrated the decreased level of ceramides in atopic skin (even in uninvolved skin) as compared with age-matched controls. Also revealed was the fact that normal-appearing aged skin was deficient in ceramide as compared with that of the younger, healthy controls. These results indicate that the decreased level of ceramides in the stratum corneum is a major etiological factor in these dry skin conditions. This finding prompted us to further investigate the mechanisms which are involved in the ceramide deficiency of atopic and aged dry skin. The synthesis of ceramides is regulated by two major enzymes, beta-glucocerebrosidase (BGCase) (8) and sphingomyelinase (SMase) (9), and the degradation mainly depends on one other enzyme, ceramidase (CDase) (10). In this study, we measured the activity of BGCase and CDase, in the stratum corneum, to clarify their association with the evolution of atopic and aged dry skin.

MATERIALS AND METHODS

Materials

4-methylumbelliferone (4-MU), 4-methylumbelliferyl (4-MU)-beta-D-glucoside (4MUG) and sodium taurodeoxycholate were obtained from Sigma Chemical Co. (St. Louis, MO).

Subjects

Sixty-three healthy volunteers (0 to 84 years old) with no abnormal skin symptoms and 23 patients with atopic dermatitis (0 to 27 years old, average age, 14.8) were included in this study. The diagnosis of atopic dermatitis was made according to Hanifin & Rajka (11). In order for us to compare BGCase activity, healthy volunteers were divided into a younger (0 to 40 years old, average age, 21.7) and an older group (41 to 84 years old, average age, 59.5). To compare healthy volunteers with patients with atopic dermatitis, we selected 23 healthy individuals (0 to 25 years old, average age, 16.1) as age-matched controls. The specimens were obtained from the normal forearm skin of healthy individuals and from uninvolved forearm skin of patients with atopic dermatitis by three tape strippings (protect label/B type, 40 × 60 mm, Iuchiseieidou Corporation, Japan) in the same region. The tapes were stored frozen at −20°C until use.

Measurement of enzyme activity

The three tapes were immersed in 40 ml of 67 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 11.6 mM sodium cholate (extraction buffer) for 30 min at 4°C, then disrupted on ice for a total of 3 min with 30-sec bursts of a sonicator (Bioruptor, Olympus Corporation, Japan). The mixture was incubated in extraction buffer overnight at 4°C to obtain a cholate extract for the subsequent assay process. After the tapes had been removed, the extract was concentrated to about 0.5 ml with a Centriprep 10 and Centricon 10 (Amicon, Millipore Corporation, USA). The concentrate was assayed for protein with BCA protein assay reagent (12) and enzyme activities of BGCase and CDase.

BGCase activity was measured using the modified method of Mier & van den Hurk (13). The assay proceeded at 37°C for 120 min in the citrate phosphate buffer (pH 5.6) with 5 mM sodium taurodeoxycholate, as the assay buffer, and 0.5 mM 4MUG. The enzyme (50 μl) in the assay buffer was heated in a plastic culture tube. The reaction was initiated by adding 50 μl of substrate in assay buffer. The reaction was terminated with 1.25 ml of 200 mM carbonate-bicarbonate buffer (pH 10.5). The fluorescence was measured (Ex = 360 nm, Em = 450 nm) with a Hitachi fluorescence spectrophotometer F-4010. A standard

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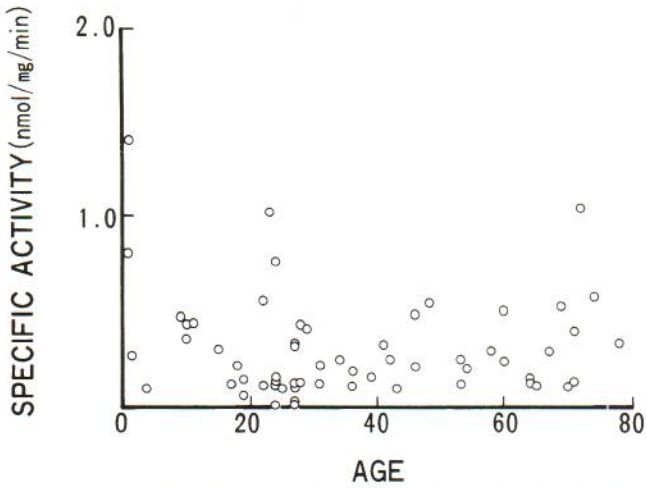

Fig. 1. BGCase activities of the stratum corneum as a function of age in healthy volunteers.

4-MU solution (0–300 nM) in carbonate-bicarbonate buffer was used for calibration.

Alkaline CDase activity was assayed by measuring the amount of radioactive palmitic acid from (1–^{14}C) palmitoylsphingosine as described previously (14). The standard mixture (final volume, 0.2 ml) contained 125 mM Tris-HCl buffer (pH 9.0), 100 μg of Tween 20, 250 μg of Triton X-100, 300 μM (1–14C) palmitoylsphingosine (34 mCi/mmol), and 150 μl of the enzymatic sample. The reaction mixture was incubated for 3 h at 37°C, and terminated with 50 μl of carrier palmitic acid (10 mg/ml in hexane), followed by 3.0 ml of Dole's reagent (2-propanol: heptane: 1N NaOH = 40: 10: 1). Heptane (1.8 ml) and 1.6 ml of water were then added. The mixture was vortex-mixed for 1 min and centrifuged for 5 min at 2,000 × g. The upper phase was aspirated, and the organic underphase was washed twice with 2 ml of heptane. Thereafter, 1 ml of 1N H_2SO_4 and 2.4 ml of heptane were added, and the mixture was vortex-mixed for 1 min, then separated by centrifugation for 10 min at 2,000 × g. A 1-ml portion of the upper phase was transferred to a vial and mixed with scintillation fluid. The radioactivity level was determined using a liquid scintillation counter.

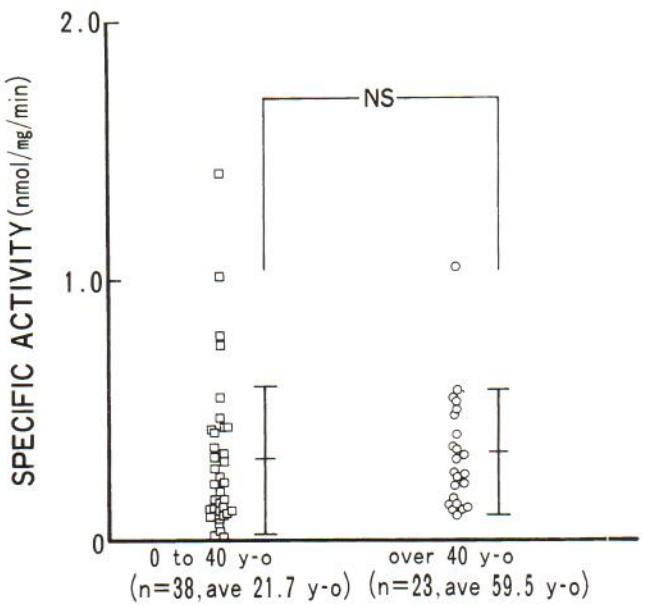

Fig. 2. A comparison of BGCase activities of the stratum corneum between healthy young and old people.

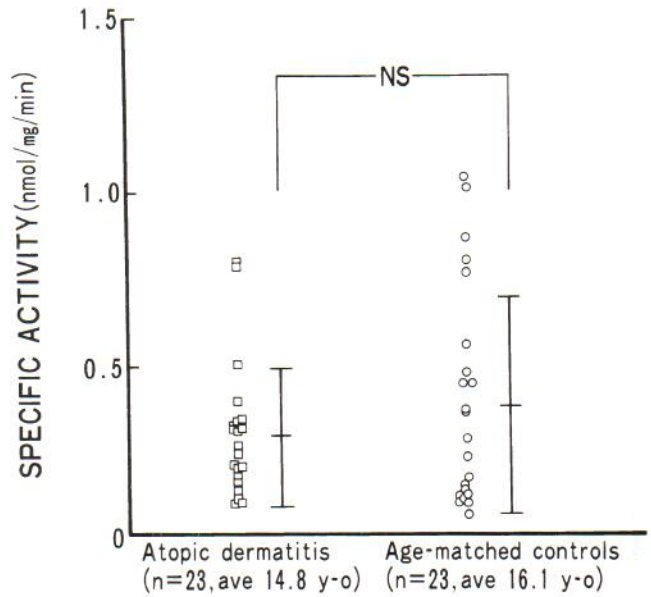

Fig. 3. A comparison of BGCase activities of the stratum corneum between atopic uninvolved skin and age-matched controls.

RESULTS

BGCase activity

Fig. 1 shows the BGCase activity (nmol/mg/min) of the stratum corneum as a function of age in healthy volunteers (n = 61). There was no significant difference in BGCase activity (mean ± SD) between younger (n = 38, average age, 21.7) and older (n = 23, average age, 59.5) groups (Fig. 2). Fig. 3 shows the BGCase activity (mean ± SD) in patients with atopic dermatitis (n = 23, average age, 14.8) and age-matched controls (n = 23, average age, 16.1). No apparent difference was observed between them, but the activity in atopic dry skin tends to be slightly higher than that in age-matched controls.

CDase activity

Since in our preliminary experiments, the activity of acid CDase was negligible in the stripped stratum corneum, we measured

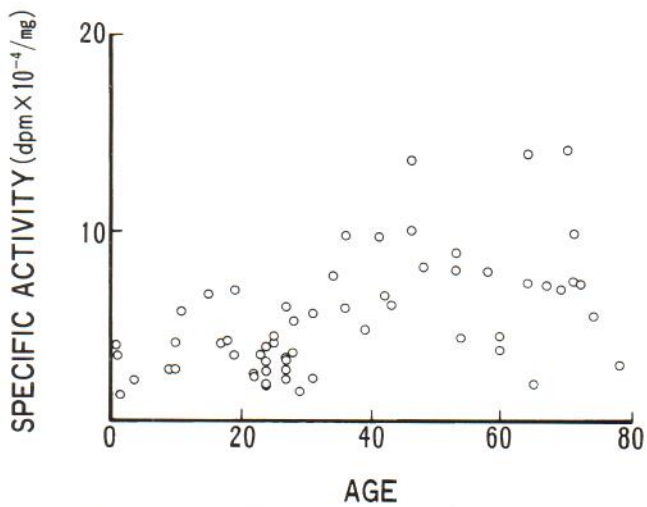

Fig. 4. CDase activities of the stratum corneum as a function of age.

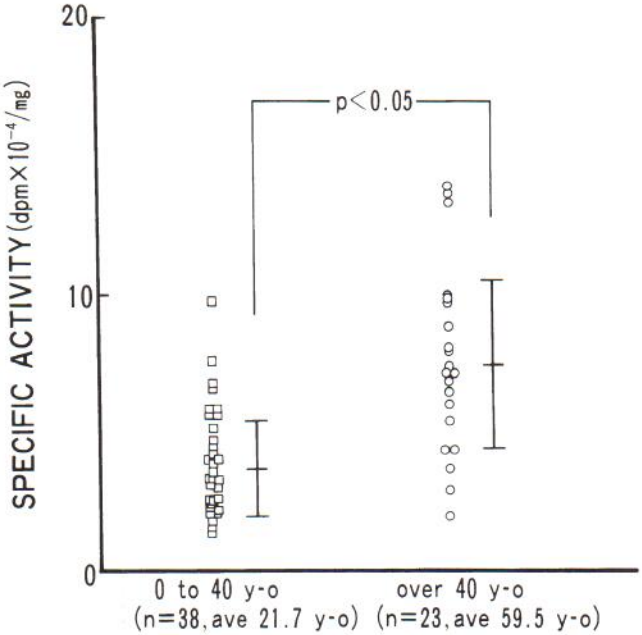

Fig. 5. A comparison of CDase levels in the stratum corneum between healthy young and old people.

alkaline CDase as a major CDase in the stratum corneum. Fig. 4 shows CDase activities (dpm × 10^{-4}) per mg stratum corneum as a function of age in healthy volunteers ($n = 61$), showing that the activities of CDase increase with age. The difference in CDase activities (mean ± SD) between younger and older healthy volunteers is statistically significant ($p < 0.05$) (Fig. 5). In contrast, the CDase activity in the uninvolved skin of patients with atopic dermatitis was not enhanced, compared with that of age-matched healthy controls (Fig. 6).

DISCUSSION

The stratum corneum lipids are composed of non-polar lipids, free fatty acids, cholesterol and cholesteryl sulfates (5). These lipids form the lamellar structure which exists between stratum corneum cells. Recent evidence has revealed the important role that these lipids play in both the water retention (15, 16) and the permeability barrier function (4, 5) of the stratum corneum. Among the lipids of the stratum corneum, ceramides are the most common constituent and account for 50% of the overall total. They are thought to play an essential role in the functions of the stratum corneum. Consequently, the ceramide composition should be associated with dry skin conditions. Among various dry skin disorders, we focused upon atopic and aged dry skin. The ceramide levels per unit mass of stratum corneum were evaluated by means of cyanoacrylate resin stripping. In atopic dermatitis, a remarkable reduction was found in the ceramide levels in both involved and uninvolved forearm skin, when compared to the levels of age-matched, healthy controls (7). Among ceramide fractions, the decrease of ceramide 1, which is considered an essential component of the barrier function, was the most prominent (7). There have been similar observations of a ceramide deficiency in atopic dermatitis (17), when ceramides have been quantified by solvent-extraction, which should be more suitable for analyzing skin surface lipids than intercellular lipids of the stratum corneum.

We have previously demonstrated a marked age-related decline in the ceramide levels of the forearm and leg skin of healthy individuals (7, 18). In senile xerosis and asteatotic eczema of the leg, frequently seen in older people in winter, a smaller decrease in ceramide levels was observed compared with that seen in age-matched healthy individuals (18). The decrease was still significant when compared with the ceramide levels of younger, healthy controls. These findings indicate that a decrease in stratum corneum lipids, especially ceramides, was a major etiological factor for atopic dry skin and a primary event in the evolution of aged dry skin. Ceramides are non-polar lipids, which are produced from the degradation of polar lipids, sphingomyelin and glycosphingolipid, which are contained in lamellar granules of spinous and granular cells. Polar lipids are secreted into the intercellular spaces around granular cells and are then decomposed in the stratum corneum by acid hydrolases. Ceramides are generated in part from sphingomyelin, as a result of being hydrolyzed by SMase, and also from glycosphingolipid through hydrolyzation by BGCase. Ceramides are concomitantly degraded by CDase. Thus, the ceramide levels are regulated mainly by the relative activity of these enzymic processes.

To further understand the mechanisms involved in ceramide deficiency in atopic and aged dry skin, we focused initially upon BGCase and CDase activities, since they are ceramide-generating and degrading factors, respectively. Their activities in the stratum corneum were evaluated by analyzing stratum corneum sheets obtained by tape stripping. Although BGCase is mainly present in lamellar granules, it was also detected in the stratum corneum and is representative of the whole epidermis (8, 19).

In atopic uninvolved skin, where the water-holding ability had already been diminished (as revealed by an examination with a capacitance conductance meter for skin surface water content), BGCase activity was not reduced and CDase activity was not enhanced when compared with the levels in age-matched healthy controls. It is thus difficult to explain the

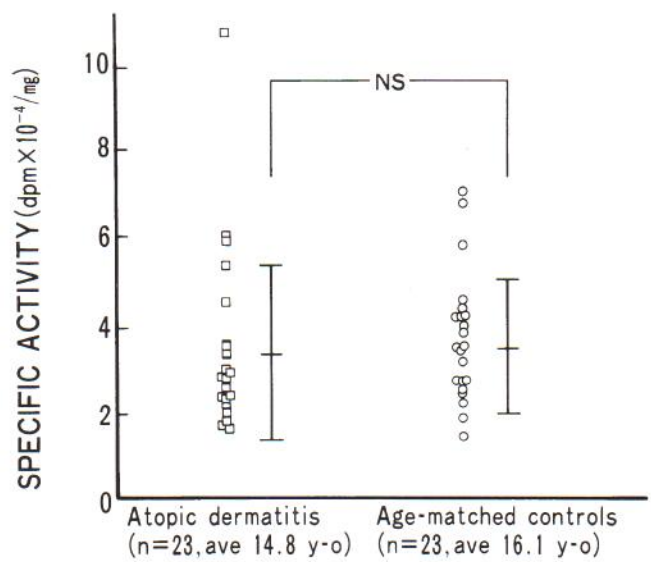

Fig. 6. A comparison of CDase levels in the stratum corneum between atopic uninvolved skin and age-matched controls.

mechanisms of ceramide deficiency in atopic dry skin only in terms of the activities of these two enzymes. This result, in fact, suggests that factors other than these enzymes are involved in the pathogenesis. One possible association is the dysfunction of several enzymes including SMase in the process of ceramide production. Whether the activity of ceramide-producing enzymes, including SMase, is altered or not in atopic dry skin remains to be clarified. Other explanations would include disturbance of the maturation of lamellar granules including ceramide synthesis, or of extrusion of lamellar granules into the spaces between stratum granulosum cells, as has been indicated by electron microscope observations (20).

In aged dry skin, BGCase activity was not less than that of younger, healthy controls. In contrast, a marked and age-related increase in CDase activity was definitely noted. This finding indicated that the rate of ceramide degradation in the stratum corneum of aged skin is significantly enhanced, which eventually results in the deterioration of the lamellar structure of the stratum corneum lipids and in evolving aged dry skin. A previous report has demonstrated the presence of only the abnormal lamellar granules (those without lamellar structures) in senile xerotic skin by electron microscopy (20). It has also been reported that SMase activity of the epidermis as a whole decreases with age (9). Given the above observations, the ceramide decrease in the stratum corneum of aged skin may well be explained in terms of the diminished synthesis associated with SMase and/or the enhanced degradation regulated by CDase.

In conclusion, disorders characterized by dry skin are divergent and it is conceivable that different pathogeneses exist. Our previous studies on two representative dry skin conditions, atopic and aged skin, demonstrated the etiological association of disorganization among the lipids of the stratum corneum, especially the ceramides (7, 18). This study, however, provided evidence that different mechanisms are involved even in similar dry skin conditions, where a decrease in the ceramide level was commonly observed.

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