Decreased Levels of Covalently Bound Ceramide Are Associated with Ultraviolet B-Induced Perturbation of the Skin Barrier

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Although ultraviolet B (UVB) irradiation perturbs the skin barrier, little is known about the mechanism(s) with respect to the metabolism of ceramide (Cer). We examined changes in intercellular lipids in murine stratum corneum following UVB irradiation. A single UVB (75 mJ per cm^2) irradiation caused a significant increase in transepidermal water loss, which plateaued at day 4. In parallel, covalently bound Cer was significantly decreased with the greatest decrease at days 3–4. In contrast, the levels of other free, non-bound lipids (including Cer or acylceramides) were significantly increased for Cer, or remained unchanged at day 4 compared with non-irradiated controls. RT-PCR analysis demonstrated a significant decrease in mRNA encoding transglutaminase-1 (TGase1). The peak occurred 2–4 d after a single UVB irradiation, a time when covalently bound Cer was significantly downregulated in concert with the disruption of the skin barrier. Furthermore, UVB-induced epidermal hyperplasia occurred to the greatest extent between 2 and 4 d following UVB irradiation. These results suggest that decreases in covalently bound Cer in the stratum corneum are mediated via the downregulation of TGase-1 as well as by the rapid induction of epidermal hyperplasia, which is attributable to the perturbation of the skin barrier induced by UVB irradiation.

Key words: ceramides/covalently bound ceramides/epidermis/permeability barrier/stratum corneum/transglutaminase/UVB irradiation
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Exposure of the skin to ultraviolet (UV) radiation induces various harmful effects in that tissue, such as hyperplasia, increases in roughness, wrinkle formation, pigmentation and inflammation (Seidl, 1963; Makki *et al*, 1979; Bissett *et al*, 1987; Imokawa *et al*, 1995), and disruption of the epidermal barrier (Abe and Mayuzumi, 1979; Solomon and Lowe, 1979; Lamaud and Schalla, 1984; McAuliffe and Blank, 1991). Epidermal ceramides (Cer) and their metabolites play critical roles in maintaining cutaneous barrier homeostasis (Elias and Friend, 1975; Gray *et al*, 1982; Elias, 1983; Landmann, 1986) and decreased levels of Cer in the stratum corneum perturb the barrier (Holleran *et al*, 1991; Imokawa *et al*, 1991). In this connection, Elias's group reported the upregulation of *de novo* Cer synthesis in murine epidermis (Holleran *et al*, 1997) and in cultured human keratinocytes (Farrell *et al*, 1998) following UVB irradiation, suggesting the possibility that the UVB-induced disruption of the skin barrier is not associated with decreased levels of Cer in the stratum corneum. Recently, Meguro *et al* (1999, 2000) demonstrated decreases in covalently bound Cer in the stratum corneum following UVB irradiation with the concomitant disintegration of lipid lamellae. Although it seems likely that covalently bound Cer rather than unbound Cer plays a role in the UVB-induced disruption of the skin barrier, no data on concentration dependence were reported and only a single time point was examined, which was not sufficient to clarify the role of covalently bound Cer in the UVB-induced barrier disruption.

Lipids in the stratum corneum comprise a distinctive mixture that is present in the intercellular domain. Most of them are easily removed by extraction with polar organic solvents (Wertz and Downing, 1986). Following such extraction, however, subsequent alkaline hydrolysis liberates additional lipids, which are bound covalently to the cornified envelope. More than half of those are hydroxyceramides that are thought to be critical for epidermal barrier function as well as corneocyte cohesion (Swartzendruber *et al*, 1987; Meguro *et al*, 1999; Behne *et al*, 2000). To clarify the relationship between the UVB irradiation-induced disruption of the cutaneous barrier and levels of covalently bound Cer in the stratum corneum, we examined sequential changes in levels of covalently bound Cer within the stratum corneum following UVB irradiation in relation to levels of other intercellular lipids, barrier disruption, and transglutaminase-1 (TGase 1) mRNA expression as well as epidermal hyperplasia.

Abbreviations: Cer, ceramide; CLE, cornified lipid envelope; FFA, free fatty acid; HPTLC, high-performance thin-layer chromatography; SDS, sodium dodecyl sulfate; TEWL, transepidermal water loss; Tgase, transglutaminase-1; UV, ultraviolet

Results

A single UVB irradiation disrupts the barrier function of the epidermis UVB irradiation at a dose of 75 mJ per cm^2 perturbed the skin barrier expressed as an increase in

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transepidermal water loss (TEWL) (Fig 1*A*). Within 12 h, the TEWL began to increase significantly and reached a plateau around day 4, which was followed by a gradual return to the unirradiated level at day 10. Significant disruption of the barrier was elicited at a dose of 37 mJ per cm^2 and increased in a concentration-dependent manner (Fig 1*B*).

Intercellular lipids in the stratum corneum It is well known that intercellular lipids play a crucial role in maintaining the barrier function of the skin. Despite the fact that the cutaneous barrier function (as evaluated by TEWL) was significantly perturbed by UVB irradiation at 75 mJ per cm^2, intercellular lipids extracted by the Bligh/Dyer method without alkali treatment were not significantly decreased in the stratum corneum at day 4 even though the TEWL had significantly increased and reached a plateau at that time (Fig 2). Although an upregulation of sphingolipid synthesis was reported following UVB irradiation (Holleran *et al*, 1997; Farrell *et al*, 1998), the UVB-induced disruption of the barrier was accompanied by significant increases in levels of free Cer and free fatty acids (FFA) in the stratum corneum, the former of which occurred in a UVB concentration-dependent manner at day 4 following UVB irradiation (Fig 3). Among those Cer, the levels of acylceramides (Cer(EOS) and Cer(EOH)), Cer(AS), and Cer(AP) were significantly increased (Fig 4 and Table I). These results support the hypothesis that among the UVB-induced alterations of lipids present in the stratum corneum, only the decrease in covalently bound Cer correlates with the increase in TEWL during UVB-induced barrier disruption.

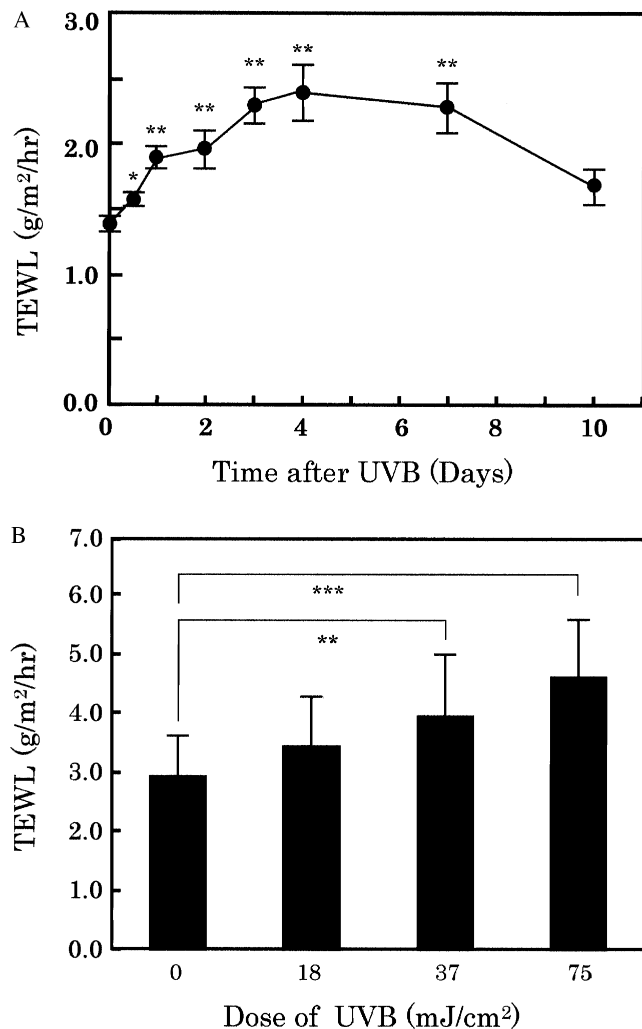

Figure 1
Changes in the cutaneous barrier function following ultraviolet B (UVB) irradiation. (*A*) Transepidermal water loss (TEWL) is increased after ultraviolet B (UVB) irradiation. Following a single dose of UVB irradiation (75 mJ per cm^2), TEWL increased with time with a peak at day 4. Mean ± SEM, n = 6, $^{*}p<0.05$; $^{**}p<0.01$ *versus* day 0. (*B*) TEWL is increased after UVB irradiation in a concentration-dependent manner. TEWL increased in a concentration-dependent manner 4 d after a single UVB irradiation. Mean ± SD, n = 4, $^{**}p<0.01$, $^{***}p<0.001$ *vs* 0 mJ per cm^2.

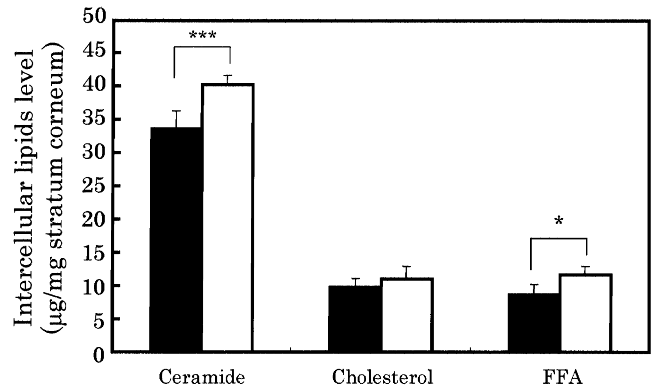

Figure 2
Unbound intercellular lipids level in the stratum corneum following ultraviolet B (UVB) irradiation. Unbound ceramide (Cer) and free fatty acid (FFA) increased significantly 4 d after a single UVB irradiation. Lipid levels were calculated by dried stratum corneum weight. Closed bar: 0 mJ per cm^2; open bar: 75 mJ per cm^2. Mean ± SD, n = 3, $^{*}p<0.05$, $^{***}p<0.001$.

Covalently bound Cer in the stratum corneum As for free Cer, covalently bound Cer may play a role in barrier homeostasis (Meguro *et al*, 2000). The level of covalently bound Cer isolated by alkaline treatment after the extraction of free Cer by the Bligh/Dyer method was significantly down-

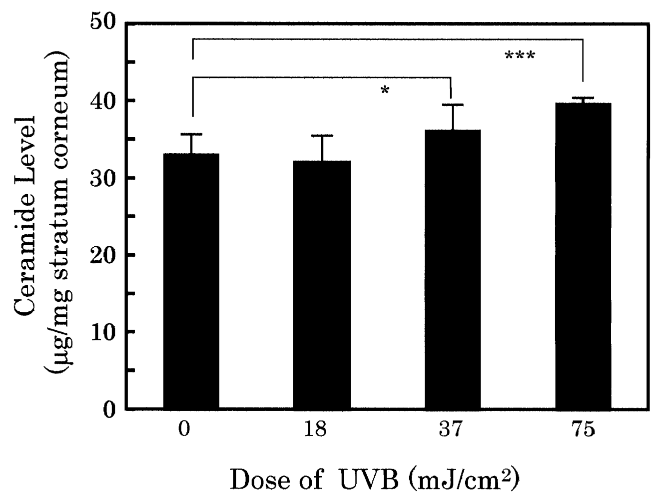

Figure 3
Unbound ceramide (Cer) levels in the stratum corneum after ultraviolet B (UVB) irradiation. UVB irradiation increased unbound Cer levels in the stratum corneum in a concentration-dependent manner on day 4. Lipid levels were calculated by dried stratum corneum weight. Mean ± SD, n = 3–8, $^{*}p<0.05$, $^{***}p<0.001$ *vs* 0 mJ per cm^2.

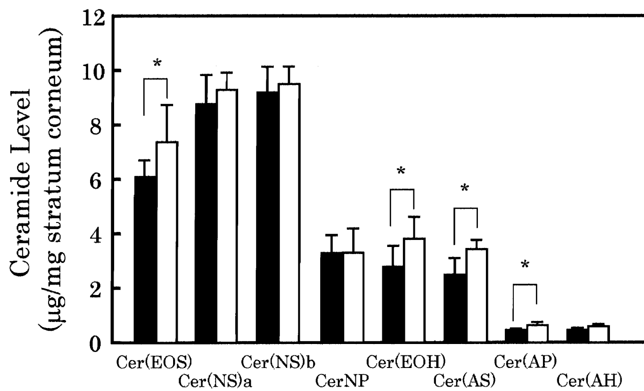

Figure 4
Acylceramide level in the stratum corneum after ultraviolet B (UVB) irradiation. Following UVB irradiation (75 mJ per cm^2), unbound ceramide (Cer) levels in the stratum corneum were analyzed at day 4. Lipid levels were calculated by dried stratum corneum weight. Mean ± SD, n = 5, $^*p < 0.05$ *vs* 0 mJ per cm^2. The Cer nomenclatures used in this figure are described in Table I.

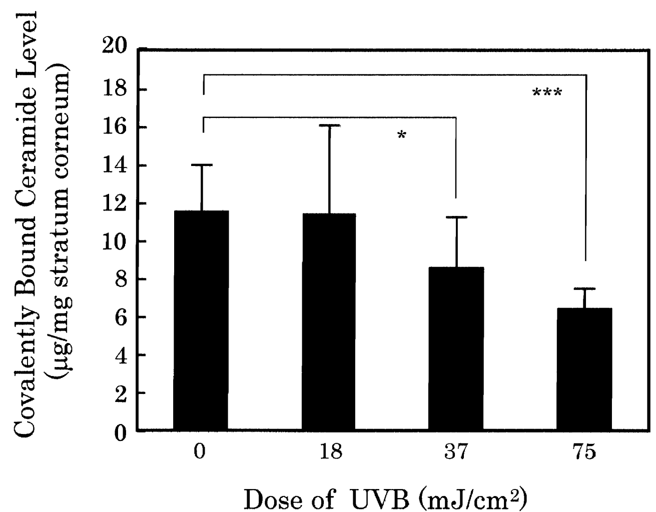

Figure 5
Covalently bound ceramide (Cer) in the stratum corneum after ultraviolet B (UVB) irradiation. The concentration-dependent effect of ultraviolet B (UVB) irradiation was examined for covalently bound Cer in the stratum corneum at day 3. UVB irradiation reduced covalently bound Cer levels in a concentration-dependent manner. Lipid levels were calculated by dried stratum corneum weight. ω-hydroxyceramide used as a standard was obtained by deacylation of acylceramide, which was purified by scraping a corresponding spot on high-performance thin-layer chromatography. Mean ± SD, n = 3, $^{**}p < 0.01$ *vs* 0 mJ per cm^2.

regulated in a concentration-dependent manner, almost to 55% of the unirradiated level at day 4 after UVB exposure at a dose of 75 mJ per cm^2 (Fig 5). Further, sphingolipids released by alkaline treatment of cornified envelopes at day 4 following a single UVB exposure included only ω-hydroxyceramide, but not ω-hydroxyglucosylceramide (Fig 6). In the UVB-exposed skin (75 mJ per cm^2), the level of covalently bound Cer in the stratum corneum was significantly decreased at days 3 and 4 following the single UVB irradiation and gradually returned to the unirradiated level by day 10 (Fig 7), a pattern that was very consistent with the increased TEWL (Fig 1*A*).

Expression of mRNA encoding TGase 1 in UVB-irradiated epidermis Since TGase 1 is thought to be associated with the attachment of ω-hydroxyceramides to involucrin by ester bond formation (Nemes *et al*, 1999), we determined the expression of TGase 1 mRNA in UVB-irradiated epidermis. Exposure of murine (BALB/c) skin to UVB at 37 mJ per cm^2 induced a significant decrease in the expression of TGase 1 mRNA transcripts at 2–4 d post-irradiation (Fig 8).

Epidermal hyperplasia following UVB irradiation It is well known that UVB irradiation induces epidermal hyperplasia (Haratake *et al*, 1997a, b). In UVB (75 mJ per cm^2)-irradiated skin, the thickness of the epidermis was significantly increased and reached a plateau at day 4 (Fig 9). This increase became quite drastic from day 2 to day 4, a time when the decrease in covalently bound Cer occurred (Fig 7).

Table I. Ceramide (Cer) nomenclatures used in this study

	Aminobond fatty acid	Sphingoid base
Cer(EOS)	ω-hydroxy fatty acid ester-linked to linoleic acid	Sphingosine
Cer(NS)	Non-hydroxy fatty acid	Sphingosine
Cer(NP)	Non-hydroxy fatty acid	Phytosphingosine
Cer(EOH)	ω-hydroxy fatty acid ester-linked to linoleic acid	6-hydroxy-4-sphingosine
Cer(AS)	α-hydroxy fatty acid	Sphingosine
Cer(AP)	α-hydroxy fatty acid	Phytosphingosine
Cer(AH)	α-hydroxy fatty acid	6-hydroxy-4-sphingosine

Tape stripping or sodium dodecyl sulfate (SDS) treatment of skin also reduces the level of covalently bound Cer To clarify the contribution of epidermal hyperplasia to the decreased level of covalently bound Cer in the stratum corneum, we determined if treatments known to induce epidermal hyperplasia (such as tape stripping or SDS washing) affect the levels of covalently bound Cer or non-bound Cer in the stratum corneum. Levels of covalently bound Cer were significantly decreased 2 d after tape stripping or SDS treatment (Fig 10), whereas the levels of non-bound Cer remained unchanged (Fig 11). These results suggest that the decreased level of bound Cer is highly associated with epidermal hyperplasia.

Discussion

In this study, a single UVB irradiation at a dose of 75 mJ per cm^2 caused a significant increase in TEWL, an indicator of the barrier function of the skin. This increase occurred in a concentration-dependent manner with a plateau at day 4 and a return to the control level by day 10. In parallel, covalently bound Cer was significantly decreased in a concentration-dependent manner with the maximum effect at days 3–4 and a return to the control level by day 7. The observed decrease in covalently bound Cer at 4 days post-

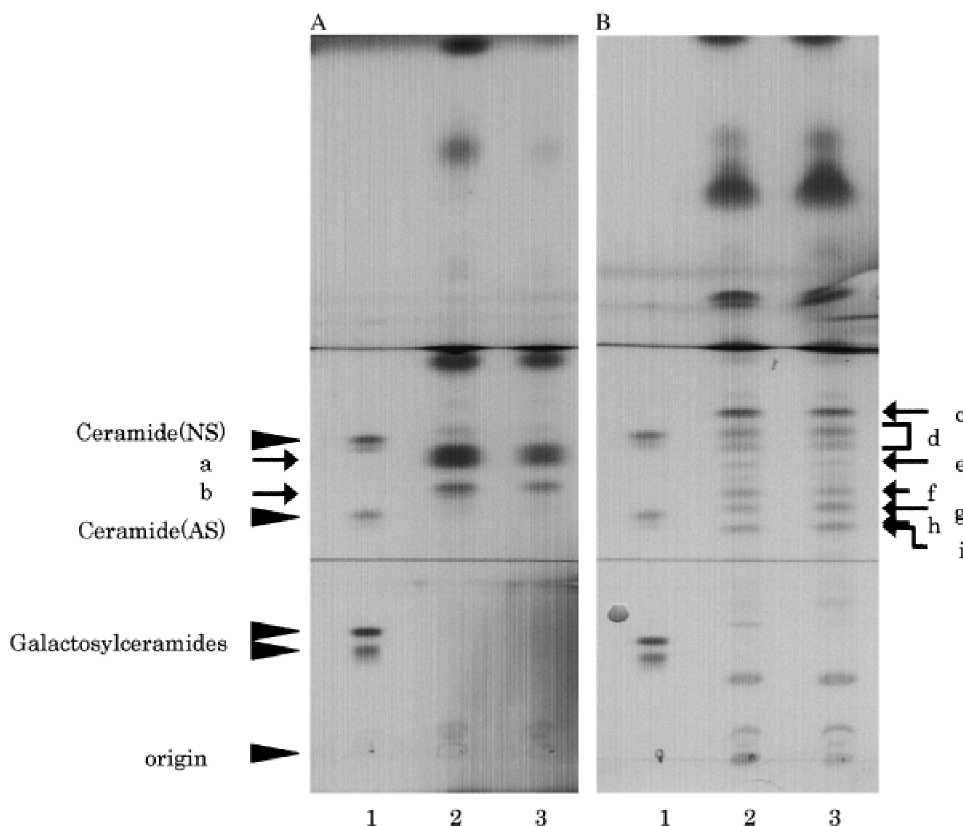

Figure 6
High-performance thin-layer liquid chromatography (HPTLC) of intercellular lipids extracted from murine stratum corneum on day 4 following UVB irradiation. Sphingolipids released by alkaline treatment from cornified envelopes following a single UVB exposure were analyzed by HPTLC. Only ω-hydroxyceramide, not ω-hydroxyglucosylceramide, covalently bound to Cer in the stratum corneum is decreased after UVB irradiation. The Cer nomenclatures used in this figure are described in Table I. (*A*) Covalently bound lipids extracted following alkali treatment. (*B*) Unbound lipids extracted by Bligh/Dyer extraction: (*1*) Standard, (*2*) 0 mJ per cm^2, (*3*) 75 mJ per cm^2. (*a*) ω-hydroxyceramide A, (*b*) ω-hydroxyceramide B, (c) Cer(EOS), (d) Cer(NS), (e) Cer(NP), (f) Cer(EOH), (g) Cer(AS), (h) Cer(AP), (i) Cer(AH).

irradiation was not associated with decreases in other unbound, free sphingolipids, but rather with significant increases in unbound, free Cer and FFA. Thus, based upon recent notions that the decreased level of intercellular lipids in the stratum corneum is primarily responsible for the aberrant barrier function of the skin (Grubauer *et al*, 1989), it seems likely that among changes in a variety of intercellular lipids, only the decrease in covalently bound Cer is paralleled by the increase in TEWL, suggesting a close association between the alteration of covalently bound Cer and the UVB-induced perturbation of the skin barrier.

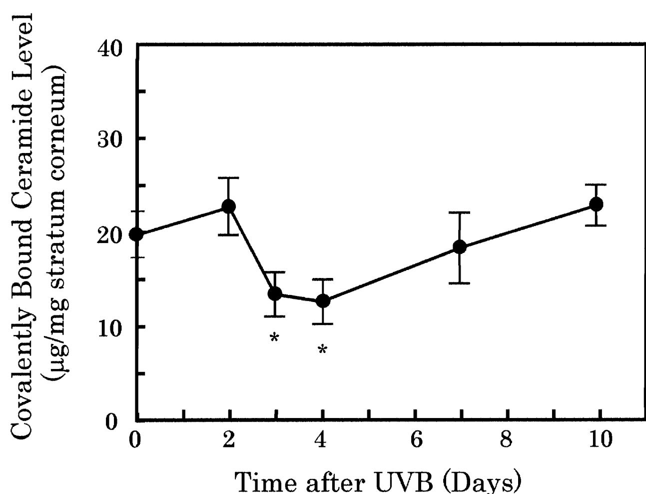

Figure 7
Covalently bound ceramide (Cer) in the stratum corneum after ultraviolet B (UVB) irradiation. Following a single dose of UVB irradiation (75 mJ per cm^2), the levels of covalently bound Cer were decreased to a maximum extent on day 3, which was consistent with the increase in transepidermal water loss. Lipid levels were calculated by dried stratum corneum weight. Mean ± SD, n = 3–5, $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* day 0.

Recently, Macheleidt *et al* (2002) reported that decreases in covalently bound Cer and in free Cer occur in atopic dermatitis as a result of downregulation of the *de novo* synthesis of Cer. But Elias's group demonstrated the upregulation of serine palmitoyltransferase (SPT) mRNA transcripts and activity in cultured human keratinocytes and the *de novo* synthesis of Cer in mouse epidermis following UVB irradiation (Holleran *et al*, 1997; Farrell *et al*, 1998). Thus, it seems reasonable to assume that the observed significant decrease in covalently bound Cer induced by UVB irradiation is mediated via biochemical mechanism(s) that are completely distinct from atopic dermatitis, where all Cer species (including covalently bound Cer) are significantly downregulated possibly due to the decreased expression of SPT.

As for the potential mechanism(s) that leads to the deficiency in covalently bound Cer in UVB-exposed skin, since UVB radiation affects the gene expression of cytochrome P-450 in keratinocytes (Wei *et al*, 1999), ω-hydroxylation via cytochrome P-450 could be inhibited directly or indirectly by UVB; this would result in decreased levels of ω-hydroxyceramides, which would in turn lead to the deficiency in covalently bound Cer. But because levels of ω-hydroxyceramide also contribute to the synthesis of acylceramide (Holleran *et al*, 1997), our results that the concomitant and significant increase in levels of acylceramide occurs in the stratum corneum of UVB-exposed skin seem to argue against the involvement of cytochrome P-450. Recent studies have revealed that ω-hydroxyceramides are ester-linked

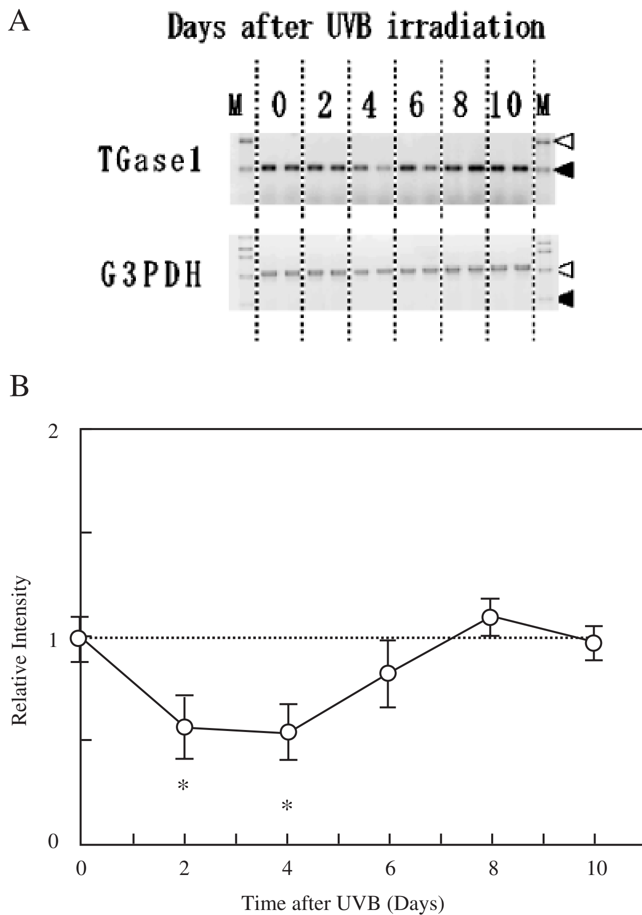

Figure 8
Analysis of transglutaminase-1 (TGase 1) expression at different times after ultraviolet B (UVB) exposure of murine skin. Shaved dorsal skin was irradiated with UVB (37 mJ per cm^2). TGase 1 mRNA expression in the epidermis was analyzed by RT-PCR at 2, 4, 6, 8, or 10 days after the irradiation. Fluorograms are shown at 30 cycles for TGase 1 and at 20 cycles for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). (*A*) M, molecular weight marker "EcoT14I-digested λDNA"; closed arrowhead, 421 bp; open arrowhead, 925 bp. The intensity of signals for PCR products was measured using fluorodensitometer. (*B*) Open circles represent the intensity for TGase 1 relative to that for G3PDH. Mean ± SD, n = 3, $^{*}p < 0.05$.

by the action of TGase 1 to glutamine and to glutamate residues of a number of cell envelope structural proteins, most notably involucrin, which serve as cornified cell envelope proteins bound to Cer and thus contribute to the barrier function of the epidermis (Marekov and Steinert, 1998; Nemes *et al*, 1999). Kuramoto *et al* (2002) have consistently reported that TGase 1 (–/–) knockout mice have perturbed skin barrier function. It has been demonstrated that TGase 1 mRNA expression is decreased after UVB-irradiation using human skin reconstituted *in vitro* (Bernerd and Asselineau, 1997). In our study, expression of TGase 1 mRNA was significantly downregulated 2–4 d after UVB-irradiation. This suggests that UVB-induced downregulation of TGase 1 may be responsible for the observed decrease in covalently bound Cer, although measurement of TGase 1 activity (which cannot be distinguished from TGase 3 activity using the standard enzymatic assay) will be required for a more precise evaluation.

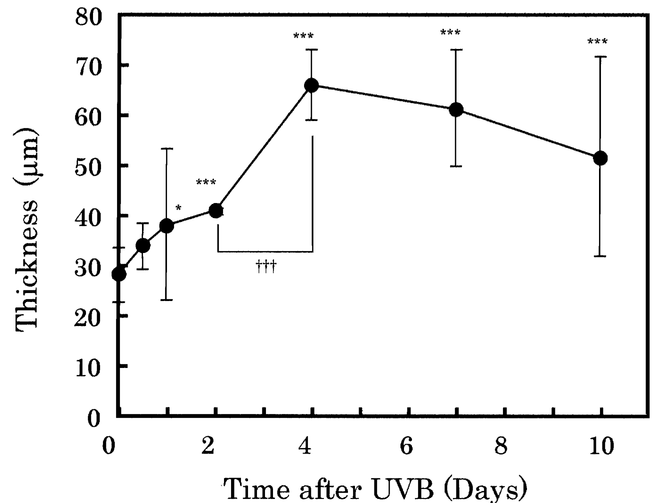

Figure 9
Epidermal hyperplasia following ultraviolet B (UVB) irradiation. Following a single dose of UVB irradiation (75 mJ per cm^2), the thickness of the epidermis was significantly increased and reached a plateau at day 4. Mean ± SD, n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* day 0.

Haratake *et al* (1997a, b) have already reported that hyperproliferation induced by UVB irradiation is associated with barrier disturbance. In our study, a similar marked epidermal hyperplasia was observed especially between days 2 and 4 following UVB irradiation in concert with a decrease in covalently bound Cer. Therefore, we attempted to determine the effects of hyperproliferation elicited by SDS treatment or by tape stripping on the level of covalently bound Cer in the stratum corneum. We found that treatments that induce hyperproliferation significantly diminished the level of covalently bound Cer, despite the lack of effect on non-bound Cer levels, suggesting the possibility that a definite relationship exists between hyperproliferation and decreased levels of covalently bound Cer. The precise mechanism(s) involved in the interrelationships among

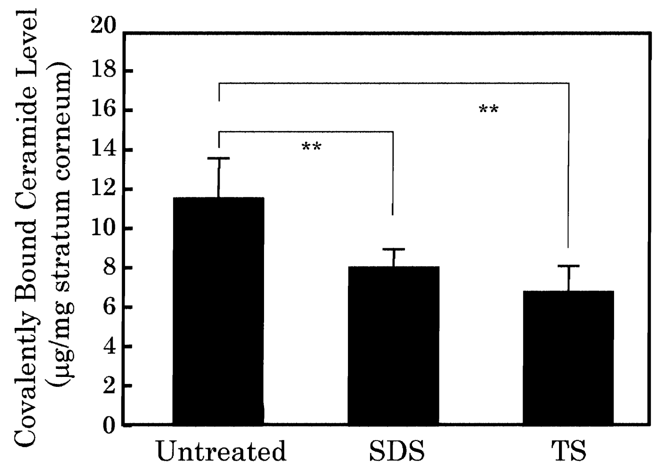

Figure 10
Decrease in covalently bound ceramide (Cer) in the stratum corneum of hyperproliferating epidermis. Covalently bound Cer decreased in the stratum corneum of hyperproliferating epidermis induced by tape stripping or by sodium dodecyl sulfate (SDS) treatment. SDS, continuous SDS treatment; TS, tape stripping. Mean ± SD, n = 3, $^{*}p < 0.05$ *versus* normal level.

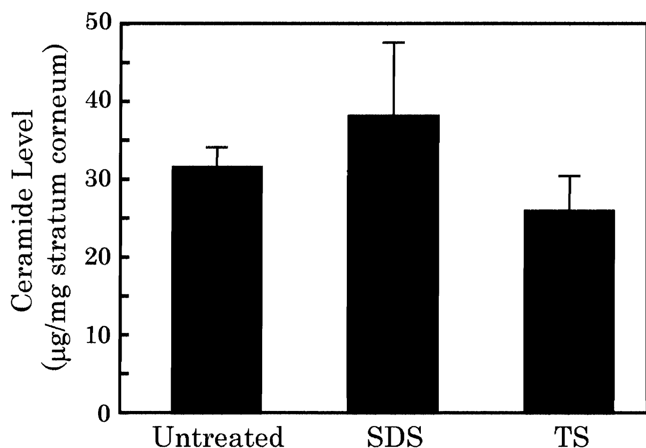

Figure 11
Ceramide (Cer) levels in hyperproliferating epidermis. Unbound Cer levels did not change in the stratum corneum of hyperproliferating epidermis induced by tape stripping or by sodium dodecyl sulfate (SDS) treatment. SDS, continuous SDS treatment; TS, tape stripping. Mean ± SD, n = 3.

hyperproliferation, decreased expression of TGase 1, and decreased levels of covalently bound Cer remain to be clarified.

As covalently bound Cer is bound to cornified envelope proteins (mainly involucrin), it is possible that UVB irradiation affects involucrin biosynthesis, resulting in the decrease in covalently bound Cer. It has, however, been reported that there is no alteration in levels of involucrin following UVB irradiation (Bernerd and Asselineau, 1997). Hirao *et al* (2001) recently studied the hydrophobicity and immunoreactivity of these components and reported that a decrease in the binding of ω-hydroxyceramides to involucrin (possibly by TGase) is elicited by UVB irradiation. These results strongly suggest that levels of covalently bound Cer could be down-regulated independent of involucrin levels.

There may be yet another mechanism that leads to the UVB-induced decrease in covalently bound Cer as follows: if ceramidase activity within the stratum corneum is stimulated by UVB irradiation and degrades covalently bound Cer, it is plausible that covalently bound Cer is also decreased in concert with an increase in covalently bound fatty acid. In our preliminary experiment, however, there was no increase in covalently bound fatty acids in the stratum corneum of UVB-exposed skin at 4 d post-irradiation, which seems to rule out a mechanism involving ceramidase.

It remains unclear whether the covalent attachment of ω-hydroxyceramide to cornified envelope proteins to form the cornified lipid envelope (CLE) occur with free ω-hydroxyceramide (the acylceramide species) and/or with ω-hydroxyglucosylceramide (Doering *et al*, 1999a, b; Behne *et al*, 2000). Since protein-bound epidermal glucosylceramides accumulate in β-glucocerebrosidase-deficient type 2 Gaucher mice (Doering *et al*, 1999b), it has been speculated that deglucosylation may normally occur following the formation of the CLE. In support of this, in the absence of β-glucocerebrosidase activity, ω-hydroxyglucosylceramide can be covalently linked to the CLE (Behne *et al*, 2000). In our study, however, we found that Cer released from the CLE by alkaline treatment following a single UVB exposure includes only ω-hydroxyceramide, not ω-hydroxyglucosylceramide. Since β-glucocerebrosidase activity is significantly diminished in the epidermis following UVB irradiation,[1] the interrupted deglucosylation of ω-hydroxyglucosylceramide or acylglucosylceramide may affect the synthesis of covalently bound Cer, although the precise roles of glucosylation and deglucosylation in the formation of the CLE remain unclear. This contrasts with the important role of glucosylation and deglucosylation in the formation of acylceramide (Takagi *et al*, 2004).

There has been speculation that acylglucosylceramide exists as a common precursor within lamellar granules (in which it serves as an assembly molecule (Landmann *et al*, 1984)) for both covalently bound Cer and acylceramide (Hamanaka *et al*, 2002). The fact that significant decreases in covalently bound Cer occur in concert with significant increases in acylceramides following UVB irradiation may be accounted for by the following mechanism: (1) the conversion of acylglucosylceramides to covalently bound Cer may be mediated via a putative pathway where a non-specific esterase is required to produce ω-hydroxyglucosylceramide, which is a substrate for TGase. (2) UVB irradiation might affect the activity of TGase to interrupt the conversion of acylglucosylceramide through ω-hydroxyglucosylceramide to covalently bound Cer. (3) This interruption may then result in the accumulation of acylglucosylceramide as well as in a preferential switch toward the biosynthesis of acylceramide, which leads to the increase in acylceramide level. Even if the pool of ω-hydroxyglucosylceramide was increased by the diminished conversion to covalently bound Cer, it is plausible that ω-hydroxyglucosylceramide generated from acylglucosylceramide by a non-specific esterase is easily converted again to acylglucosylceramide by acyltransferase.

It seems reasonable to assume that several factors other than the decreased levels of covalently bound Cer can contribute to the UVB-induced barrier perturbation. Thus, we have recently found that the decrease in β-glucocerebrosidase enzymatic activity in the epidermis was also significantly reduced by UVB irradiation, which corresponded to a significant increase in TEWL at days 1–2 post-irradiation.[1] Nevertheless, our results collectively suggest the possibility that the decreased levels of covalently bound Cer in the stratum corneum are mediated via epidermal hyperplasia and decreased expression of TGase 1, and are attributable to the perturbation of the skin barrier induced by UVB irradiation.

Materials and Methods

Animals Hairless male mice (Hr/Hr) or female BALB/c mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). All animals were 8–12-wk old at the time of study. A single dose of UVB (290–320 nm) was administered using a Toshiba FL20S · E sunlamp (Toshiba, Tokyo, Japan) to shaved or unshaved dorsal skin. Transepidermal water loss (TEWL) was measured with an electrolytic water analyzer (Meeco, Warrington, Pennsylvania) to characterize epidermal barrier function.

[1]Takagi Y, Nakagawa H, Yaginuma T, Takema Y, Imokawa G. UVB irradiation decrease β-glucocerebrosidase activity in murine epidermis. 2004, In preparation.

Table II. Oligonucleotides used for PCR analysis in this study

Molecule	Primer	Sequence (5′–3′)	Product size (bp)	Reference
TGase1	5′	TGAATAGTGACAAGGTGTACTGGCA	524	Phillips *et al* (1990)
	3′	GTGGCCTGAGACATTGAGCAGCAT		
G3PDH	5′	TGAAGGTCGGTGTGAACGGATTTGGC	983	Sabath *et al* (1990)
	3′	CATGTAGGCCATGAGGTCCACCAC		

TGase1, transglutaminase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

Extraction of lipids from the stratum corneum Unbound lipids were extracted by a modification of the Bligh/Dyer method (Bligh and Dyer, 1959) as follows. Skin samples were excised from the back of euthanized mice (Hr/Hr) and the subcutaneous tissue was removed by scraping with a scalpel blade, as described previously (Holleran *et al*, 1992). Epidermal sheets were obtained from the skin samples by exposure to dry heat (60°C for 60 s) and gentle scraping with a scalpel blade. Stratum corneum sheets were obtained from epidermal sheets by trypsinization (0.5% in phosphate-buffered saline (PBS) without calcium or magnesium, pH 7.4) at 37°C for 1 h. Following freeze drying and weighing, tissues were soaked in Bligh/Dyer solution (chloroform:methanol:H_2O = 2:4:1.6 mL) for 16 h at room temperature and then homogenized using a glass homogenizer. After removal of residue by centrifugation, 2 mL chloroform and 2 mL H_2O were added, and the lower phase was concentrated by nitrogen gas and served as the unbound lipid sample. The removed residue of stratum corneum was subsequently saponified in 500 mL 1 N NaOH in 90% methanol at 80°C for 2 h to release the lipids covalently bound to the stratum corneum by ester-like bonds (Serizawa *et al*, 1993). Following removal of the residue by centrifugation and neutralization with HCl, 2.5 mL chloroform and 2.5 mL H_2O were added and the mixture was shaken vigorously. The lower phase was concentrated by nitrogen gas and served as the ester-bound lipid sample.

High-performance thin-layer chromatography (HPTLC) analysis The dried samples were re-dissolved in chloroform/methanol (2/1) solvent and fractionated by HPTLC on 20 × 10 cm plates (Silica gel 60, Merck, Darmstadt, Germany). Cers were developed twice over 9 cm with chloroform/methanol/acetic acid (190:9:1 vol) followed over 2.5 cm with chloroform/methanol/acetone (76:20:4 vol) (Ponec *et al*, 1988; Holleran *et al*, 1993). Cholesterol and FFA were separated with hexane/ether/acetic acid (80:20:2 vol). After the final solvent run, the dried plates were dipped in acetic acid/sulfuric acid/H_2O (50:10:95 vol) and were charred at 180°C for 15 min. The plates were scanned and analyzed using a Densitometer (Lane Spot Analyzer, Atto, Japan). Cer (NS) and Cer (AS) was used as standard Cer, and palmitic acid was used as the standard FFA.

RT-PCR analysis of mRNA transcripts for TGase 1 Murine (BALB/c) skin was excised and incubated in PBS containing 2 M NaBr at room temperature for 30 min, after which the epidermis was peeled away from the dermis. Total cellular RNAs were prepared from the epidermis by the standard acid guanidium phenol–chloroform method (Chomczynski and Sacci, 1987). The epidermis was homogenized with a manual plastic homogenizer. The RNAs were analyzed for specific gene expression by RT-PCR using an RNA PCR Kit (Takara Shuzo Co., Osaka, Japan) and oligo $d(T)_{18}$ (New England Biolabs, Inc., Beverley, Massachusetts). For PCR, a total of 20–35 cycles was used; each cycle included denaturation for 1 min at 94°C, re-annealing for 2 min at 65°C and extension for 1.5 min at 72°C. PCR primers used in this study are summarized in Table II. PCR products were analyzed with a digital fluorodensitometer (FM-BIO100, Hitachi Software Engineering Co., Kanagawa, Japan) after agarose gel electrophoresis and ethidium bromide staining. All RNA samples were tested for integrity and quantity by RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Table II). For quantitative precision, 40 ng of total RNA was consistently used for each expression analysis, and its amplification kinetics were monitored for PCR by examining aliquots of the products on gels. Amounts of the PCR products were compared during the cycle where the logarithmic amplification was attained before reaching saturation (Kondo *et al*, 1994). In each electrophoresis run, intra- and inter-gel staining homogeneity was confirmed by staining intensity of molecular weight markers at both ends of the gels.

Epidermal hyperplasia The effects of UVB irradiation on epidermal hyperplasia were evaluated by measuring the thickness of the epidermis, excluding the stratum corneum. Murine dorsal skin was biopsied, fixed in 10% formaldehyde, and stained with hematoxylin and eosin to measure the epidermal thickness. The thickness of the Malpighian layer of the epidermis, namely the distance from the dermis/epidermis interface to the junction between the granular and the keratin layers, was measured microscopically at more than 200 sites per section using a Leica Q500 microscope (Leica Microsystems AG, Wetzlar, Germany).

Other treatments

Cumulative SDS treatment Murine (Hr/Hr) dorsal skin was gently rubbed once a minute with a cotton ball soaked in 2% SDS solution for 5 min, and then rinsed off with distilled water. Following 4 continuous days of treatment, intercellular lipids in the stratum corneum were analyzed as described above.

Tape stripping The stratum corneum was removed from murine (Hr/Hr) dorsal skin by repeated application of adhesive tape (CP-10, Nichiban Co. Ltd, Tokyo, Japan). Four days later, intercellular lipids level was analyzed as described above.

Statistical analysis A non-parametric one-way ANOVA (Kruskal–Wallis) test was used to evaluate differences between groups. Where appropriate, a non-parametric *post hoc* multiple comparison test (Steel–Dwass test) was performed to evaluate differences between the groups. A p value < 0.05 was considered statistically significant.

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