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Th1 cytokines accentuate but Th2 cytokines attenuate ceramide production in the stratum corneum of human epidermal equivalents: An implication for the disrupted barrier mechanism in atopic dermatitis

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ABSTRACT

Background: Although the mechanism(s) involved in the ceramide deficiency in the stratum corneum (SC) of atopic dermatitis (AD) skin is unknown, Th2 type cytokines have been reported to down-regulate ceramide levels in the epidermis. However, almost all research to date has focused on ceramide levels in the whole epidermis, not just in the SC layers alone, which are predominantly responsible for the skin barrier function.

Objective: We highlighted the effects of Th1/Th2 cytokines on ceramide levels in the SC.

Methods: We developed a modified system of human epidermal equivalents in which epidermis without a SC is cultured for 1 week to generate complete SC layers after which ceramides are extracted from the separated SC layers and are then quantified as per SC protein.

Results: The addition of Th2 cytokines (IL-4/IL-6) at a concentration of 10 nM resulted in a marked decrease in SC ceramide levels. The reduced ceramide content in the SC was accompanied by the down-regulated expression of the genes encoding serine-palmitoyl transferase-2, acid sphingomyelinase and β -glucocerebrosidase in the epidermis. In contrast, the addition of Th1 cytokines (GM-CSF/IFN- γ /TNF- α) at concentrations of 2.5 or 10 nM resulted in a slight increase in SC ceramide levels, which were accompanied by no change or an increase in the expression of those genes encoding sphingolipid metabolic enzymes in the epidermis.

Conclusion: These findings suggest that the Th2 type of inflammation evoked in AD skin is one of the essential factors involved in down-regulating the levels of ceramide in the SC.

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1. Introduction

Atopic dermatitis (AD) is characterized clinically by the presence of atopic dry skin and pathophysiologically by cutaneous barrier disruption in the stratum corneum (SC), which causes itchy and irritant or allergen-susceptible skin, leading to recurrent dermatitis. We previously reported that the barrier disrupted dry skin is mainly attributable to significantly decreased levels of ceramides in the SC [1], which act as a water modulator [2] and a permeability barrier by forming multi-layered lamellar structures

with other lipids between cells in the SC layers. The importance of the deficit in ceramides in the SC to disrupt the cutaneous permeability barrier in AD was supported by the facts that trans-epidermal water loss (TEWL) measured in AD skin increases inversely with the decreased levels of ceramides in the SC from the same skin site of AD patients [3,4] and that impaired barrier function which occurs in AD skin or is induced by surfactant or solvent treatment can be repaired by topical application of ceramides [5–7].

Since ceramide levels in the SC are biochemically regulated by the balance of 3 sphingolipid hydrolysis enzymes, β -glucocerebrosidase (BGCse), acid sphingomyelinase (aSMase) and acid ceramidase (aCDase), it was important to determine whether those enzymes are down- or up-regulated in activity, leading to the ceramide deficiency in the SC of patients with AD. Those 3 sphingolipid hydrolysis enzymes are not down-regulated at the activity level [8–10] or at the protein level [11] in the epidermis or

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the SC from AD skin, although one study reported the decreased activity of aSMase [12]. We then discovered a novel enzyme, glucosylceramide sphingomyelin deacylase (GSDase), which cleaves the N-acyl linkage of sphingomyelin and glucosylceramide [9,13]. The enzymatic activity of GSDase was significantly up-regulated in AD lesional epidermis as well as in the involved and uninvolved SC of AD skin, but not of contact dermatitis or chronic eczema, compared with healthy controls [10,14]. GSDase hydrolyzes sphingomyelin or glucosylceramide to yield sphingosylphosphorylcholine or glucosylsphingosine, respectively, instead of ceramide, which leads to the ceramide deficiency. Consistent with the increased activity of GSDase in AD skin, those hydrolyzed products (sphingosylphosphorylcholine and glucosylsphingosine) accumulate to a greater extent in the involved and uninvolved SC of AD skin compared with chronic eczema or contact dermatitis as well as healthy controls [14,15]. Recently, GSDase has been purified to homogeneity from rat skin and was identified as the β -subunit of aCDase, which consists of α - and β -subunits linked by two S–S bonds.² In fact, breaking those S–S bonds by dithiothreitol elicited the activity of sphingomyelin deacylase.² Although the highly elevated activity of GSDase in AD skin seems to play a key role in the ceramide deficiency, it remains unclear why the β -subunit of aCDase is released at the interface between the SC and stratum granulosum layers in AD skin.

It has been documented that T helper (Th)2- and Th1-type immunology plays an important pathogenic role in the acute phase and the chronic phase, respectively, of AD skin [16,17]. The high levels of IgE in the blood and the infiltration of eosinophils into the inflammatory tissue of the skin are the major pathophysiological characteristics for AD patients and are mainly attributable to Th2-type inflammatory events in which there is a high secretion of Th2-type cytokines such as IL-4 and IL-6 [17,18]. Atopic dermatitis represents a hyper-reactive condition in response to a typical Th2 cytokine IL-4 [19]. Since we have found that if percutaneous sensitization is performed in barrier-disrupted skin [20], a Th2-dominant reaction is evoked in the afferent phase, it remains to be clarified whether the immunological alteration is a causative factor for the barrier disruption or is a result of the barrier deficiency. As for the mechanism(s) involved in the ceramide deficiency in the SC of AD skin, it has recently been reported that Th2-type cytokines affect the expression of ceramide-producing enzymes, which result in the reduced levels of ceramide in the epidermis accompanied by the disrupted barrier function [21]. In addition, IL-4 blocks the recovery of barrier function and the enhancement of ceramide synthesis after barrier disruption by acetone treatment of living skin equivalents [22]. Although the study of Hatano et al. [22] provides an interesting insight into the mechanism involved in the ceramide deficiency in AD, all studies to date have focused on ceramide levels in the whole epidermis but not in the SC layers alone, which are predominantly responsible for the cutaneous barrier function. Also, that study reported only changes in the expression of genes encoding the 3 ceramide metabolic enzymes (BGCase, aSMase and aCDase) in the presence of IL-4 and INF γ . Thus, the lack of Th1/Th2 cytokine effects on the SC ceramide levels as well as protein and enzymatic activity levels of the 3 ceramide metabolic enzymes hampers our understanding of the precise effects of the Th1/Th2 cytokine balance on ceramide metabolism in the SC.

To precisely elucidate the effects of the Th1/Th2 cytokine balance on ceramide levels in the SC as well as on the expression of ceramide metabolic enzymes (CME) at the gene, protein and enzymatic activity levels in the epidermis, we developed an

advanced system in which 3-dimensional epidermal sheets (consisting of multi-layered foreskin-derived human keratinocytes) without the SC layers under pre-keratinizing conditions were cultured for 1 week to generate complete SC layers, which could then be separated and ceramides extracted and quantified as per SC protein [23]. Comparison of the lipid composition by thin layer chromatography (TLC) shows that the pattern of lipids obtained from the whole SC layers of the 3-dimensional epidermal model is almost identical to that from the 3 SC layers of normal human forearm skin [23], indicating the suitability of the 3-dimensional epidermal model. Our recent study using inhibitors of ceramide-producing enzymes in the same epidermal model indicated that the major metabolic pathways leading to ceramide synthesis through the interface between the SC and the granular layer occur in the 3-dimensional epidermal models in a pattern similar to that seen in vivo [23]. Here we report that the Th2-type of inflammation evoked in AD skin is one of the essential factors in down-regulating the level of ceramides in the SC.

2. Materials and methods

2.1. Materials

4-Methylumbelliferone (4-MU), 4-methylumbelliferyl- β -D-glucopyranoside (4MUG), and sodium taurodeoxycholate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The BioRad Protein Assay kit and bovine serum albumin (BSA) were obtained from BioRad Laboratories (Richmond, CA, USA). Human recombinant GM-CSF (215-GM), human recombinant IFN- γ (285-IF), human recombinant IL-8 (208-IL), human recombinant IL-4 (204-IL), human recombinant IL-6 (206-IL) and human recombinant TNF- α (210-TA) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

2.2. Reconstructed human epidermal keratinization model

The reconstructed human epidermal keratinization (RHEK) model consists of multilayered foreskin-derived human keratinocytes (LabCyte EPI-MODEL, Japan Tissue Engineering Co., Ltd.), established by Green's method [24,25], which are prepared under pre-keratinizing conditions prior to formation of the SC layers. RHEK models were cultured at 37 °C for 7 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5 μ g/ml hydrocortisone, 5 ng/ml EGF, 5% fetal bovine serum (FBS) and 1.5 mM CaCl₂, containing several cytokines or PBS as the control. The medium was replaced every day. After the indicated days of culture, tissues were peeled from the membranes and were subjected to lipid analysis, real-time RT-PCR analysis, western blotting and histochemical staining as detailed below.

2.3. Lipid extraction

After culture, the whole RHEK model was peeled from the membrane and the epidermal cells were separated from the SC by incubation in a trypsin/EDTA aqueous solution at 37 °C for 1 or more hour. The separation was confirmed by microscopic observation. Both the SC and the epidermal cell samples were washed with PBS and were stored at –80 °C until used. Lipids were extracted using a modification of the Bligh/Dyer method [26]. Thus, both the SC and the epidermal cell samples were homogenized in chloroform:methanol:PBS (1:2:0.8 vol) and were then mixed for 20 min following centrifugation at 3000 rpm for 15 min. Each supernatant was collected in a glass test tube and 1 mL chloroform and PBS were added. After 20 min mixing and centrifugation (3000 rpm, 15 min), the bottom layer was collected and dried at 30 °C under nitrogen gas.

² Nogami-Itoh M, Teranishi Y, Kuwahara H, Kusumoto M, Nakamura K, Matsumoto M, et al. Purification and identification of sphingomyelin deacylase from rat skin. *J Invest Dermatol* 2010;13 (Suppl. 2):s24.

2.4. Thin layer chromatography

The amounts of ceramides were determined by high performance thin layer chromatography (HPTLC) as described by Imokawa et al. [1]. The dried samples were dissolved in chloroform/methanol (2/1) solvent and were fractionated by HPTLC on 20 × 10 cm silica gel plates (Merck KGaA, Darmstadt, Germany). Ceramides were developed twice over 9 cm with chloroform/methanol/acetic acid (190:9:1 vol). After the final solvent run, the dried plates were sprayed with 10% copper sulfate in 8% phosphoric acid aqueous solution and were charred at 180 °C for 7 min. The plates were scanned and analyzed using Quantity One (BioRad Laboratories). Ceramide reagents (Matreya, Pleasant Gap, PA, USA) were used as standards.

2.5. Real time RT-PCR

The expression of mRNAs encoding serine-palmitoyl transferases (SPT-1 and -2), aSMase, BGCCase and aCDase was characterized by quantitative realtime RT-PCR. mRNA was extracted from each whole RHEK model using an RNeasy mini kit (QIAGEN, Tokyo, Japan) followed by reverse-transcription using Revertra Ace Kit (Toyobo, Tokyo, Japan). The specific primers used for realtime PCR are as follows; aSMase, 5'-TGGCTCTATGAAGCGATGG-3' as upstream and 5'-AGGCCGATGTAGGTAGTTGC-3' as downstream; aCDase, 5'-CCTTCTCTCTTGATGATCGC-3' as upstream and 5'-GTGGAGTTCACCATGGTTCCG-3' as downstream; SPT-1, 5'-GCGCGCTACTTGGAGAAAGA-3' as upstream and 5'-TGTTCCACCGTGACCACAAC-3' as downstream; SPT-2, 5'-AGCCGCCAAAGTCCTTGAG-3' as upstream and 5'-CTGTCCAGGTTTCAATTTCC-3' as downstream; aSMase, 5'-TGGCTCTATGAAGCGATGG-3' as upstream and 5'-AGGCCGATGTAGGTAGTTGC-3' as downstream; BGCCase, 5'-TGGCATTGCTGTACATTGG-3' as upstream and 5'-CGTTCTTCTGACTGGCAACC-3' as downstream.

2.6. Western blotting

Each RHEK model was lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl and 1% Triton X-100) plus protease inhibitors (Complete mini-tablets; Boehringer Mannheim, Mannheim, Germany), subjected to ultrasonication and centrifuged at 18,000 × g for 15 min. The supernatants were harvested and lysed in loading buffer (125 mM Tris, pH 6.8, 4.6% sodium dodecyl sulfate (SDS), 20% glycerol and 0.04% pyronin Y), separated on 10% SDS-PAGE gels, and blotted to PVDF membranes (BioRad Laboratories). After blocking with 5% skim milk, the membranes were treated overnight at room temperature with primary antibodies as follows: rabbit anti-human ASAH1 polyclonal antibody (Protein-tech Group, Chicago, IL 60612, USA) for aCDase, rabbit anti-acid sphingomyelinase polyclonal antibody (Exalpha Biologicals, Shirley, MA, USA) for aSMase, and mouse monoclonal anti-beta-actin clone ac-74 ascites fluid (Sigma) for β-actin. The membranes were treated at room temperature for 1 h with secondary antibodies as follows: anti-rabbit IgG, HRP-linked whole Ab donkey (GE Healthcare, Buckinghamshire, UK) for aCDase and aSMase, and anti-mouse IgG, HRP-linked whole Ab sheep (GE Healthcare) for β-actin. The bands were detected by treatment with ECL reagents followed by exposure to X-ray film. The film was scanned and analyzed using Quantity One software (BioRad Laboratories).

2.7. Protein analysis of the stratum corneum and epidermal cells

Amounts of protein in SC or epidermal cell samples were quantified and used for normalization of ceramide levels. The precipitates obtained after extracting the lipids were dissolved in 1% SDS/1 M NaOH at 60 °C for 2 h. After neutralization with HCl,

the total protein amounts were analyzed using a BCA protein assay kit (Thermo Scientific) according to the manufacturer's protocol.

2.8. Preparation of enzyme solution

Each RHEK model was homogenized with a Polytron PCU2 Tissue Homogenizer (15 s × 3, Kinematica AG, Lucerne, Switzerland), in 0.3 ml of 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100 and 0.5 mM PMSF, followed by sonication (10 W, 10 s × 2) using an Ultrasonic Generator (Cho-onpa Kogyo, Tokyo, Japan) for 5 min on ice. Cleared supernatants after the removal of the stratum corneum and cell residues by centrifugation (10,000 × g, 30 min) were used as the enzyme solution. Protein concentrations were measured using the BioRad Protein Assay Kit with BSA as the standard.

2.9. Assay of BGCCase activity

Enzyme assays were carried out as described previously [27]. Briefly, assays were carried out at 37 °C in citrate–phosphate buffer (pH 5.6) with 0.5 mM sodium taurodeoxycholate as the assay buffer and 0.5 mM 4MUG as the substrate. Reactions were initiated by the addition of 50 μl substrate solution and 50 μl enzyme solution in 50 μl assay buffer. After 60 min, the reactions were terminated by the addition of 1.25 ml 200 mM carbonate–bicarbonate buffer (pH 10.5). The fluorescence was measured (Ex = 360 nm, Em = 450 nm) with a spectrofluorimeter (Hitachi 3000, Tokyo, Japan). A standard 4-MU solution (0–300 nM) in carbonate–bicarbonate buffer was used for calibration. BGCCase activity is expressed as nmol/min/mg protein.

2.10. Assay of a SMase activity

Acid SMase activity was measured according to the method of Van Diggelen et al. [28]. Reaction mixtures consisted of 10 μl enzyme solution, 10 μl 1.35 mM 6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine (HMU-PC) (Moscerdam, Oegstgeest, Netherlands) as a substrate in 0.1 M Na-acetate/0.1 M acetic acid buffer, pH 5.2, containing 0.2% (w/v) Na-taurocholate (Sigma). The reaction mixtures were incubated for 1 h at 37 °C. Reactions were terminated by the addition of 200 μl stop buffer containing 0.5 M NaHCO₃/0.5 M Na₂CO₃, pH 10.7, and 0.25% Triton X-100. The fluorescence of 6-hexadecanoyl-4-methylumbelliferone (HMU) was measured on a fluorimeter (FluoroCount; Packard), using the filter set of 4-methylumbelliferone (MU): excitation = 360 nm, emission = 460 nm. The fluorimeter was calibrated with MU in stop buffer. aSMase activity was expressed as nmol/h/mg protein.

2.11. Statistics

All data are expressed as means ± SD (n = 3 or 6) unless noted otherwise. For pairwise comparisons, the Student's *t* test was applied. Only *p* values less than 0.05 which are considered statistically significant were displayed in each figure.

3. Results

3.1. Effects of GM-CSF on SC or epidermis ceramide levels and sphingolipid metabolic enzymes at the gene, protein and enzymatic activity levels

Addition of the Th1 cytokine, GM-CSF, at a concentration of 10 nM during the 7 days of RHEK model in culture induced no degenerative changes in the epidermis (Fig. 1). TLC analysis for lipids isolated from the stratum corneum (Fig. 2) revealed that GM-CSF significantly increased ceramide levels expressed as

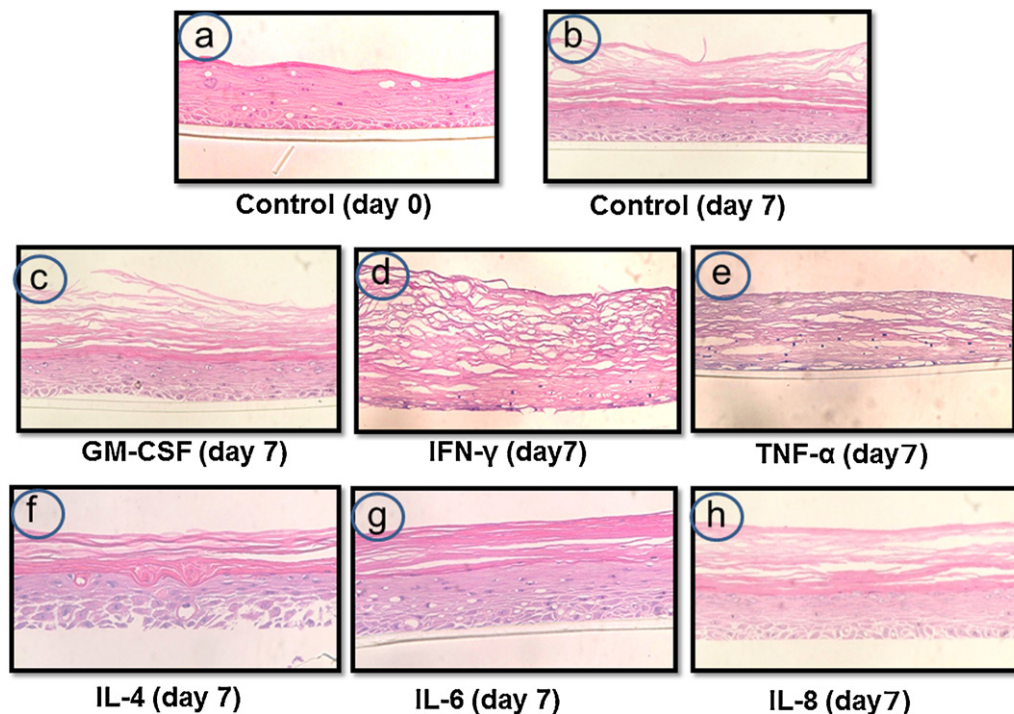


Fig. 1. Effects of cytokines on the epidermis as evaluated by H&E staining. a: control (day 0), b: control (day 7), c: GM-CSF (day 7), d: IFN- γ (day 7), e: TNF- α (day 7), f: IL-4 (day 7), g: IL-6 (day 7), h: IL-8 (day 7). RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN- γ) and subjected to H&E staining.

$\mu\text{g}/\text{well}$ or $\mu\text{g}/\text{mg}$ protein in the SC (Fig. 3). On the other hand, TLC analysis for lipids isolated from the epidermis (Fig. 2) revealed that there was no significant difference in ceramide levels expressed as $\mu\text{g}/\text{well}$ or $\mu\text{g}/\text{mg}$ protein in the epidermis excluding the SC between GM-CSF treated and non-treated RHEK models (Fig. 3). Composition analysis of ceramide species demonstrated that among all ceramide species of the SC, CERs [NDS/NS] and [NH] were significantly increased by GM-CSF treatment (Table 1), whereas there was no significant difference in all ceramide species of the epidermis between GM-CSF treated and non-treated RHEK models (Table 2). There was no significant difference between the GM-CSF-treated and the non-treated RHEK models in the expression of the genes encoding the initial enzyme in sphingolipid metabolism, SPT-1 or SPT-2 at day 4 of culture (Fig. 4). Also there was no significant difference in the expression of genes encoding the ceramide-producing enzymes aSMase or BGCase or the degrading enzyme aCDase at day 4 of culture between the GM-CSF-treated and the non-treated RHEK models (Fig. 4). In contrast, the treatment with GM-CSF at 10 nM significantly stimulated the protein level of aSMase (Fig. 5) but not aCDase (Fig. 6) and significantly increased the enzymatic activity of both aSMase and BGCase at day 7 of culture (Fig. 7). The sum of these findings indicates that GM-CSF significantly increases ceramide/protein level in the SC by up-regulating both aSMase and BGCase at the post-transcriptional level.

3.2. Effects of IFN- γ on SC ceramide levels and sphingolipid metabolic enzymes at the gene, protein and enzymatic activity levels

Since the effect of another TH1 cytokine, IFN- γ , at a concentration of 10 nM on the epidermal tissue was found to be harmful, we used a concentration of 2.5 nM. The addition of IFN- γ at 2.5 nM induced a marked hyper-keratinizing change in the epidermis (Fig. 1) and resulted in a slight but not significant increase of ceramide level (expressed as $\mu\text{g}/\text{mg}$ protein of the SC) as a result of significant decreases in ceramide/well and in

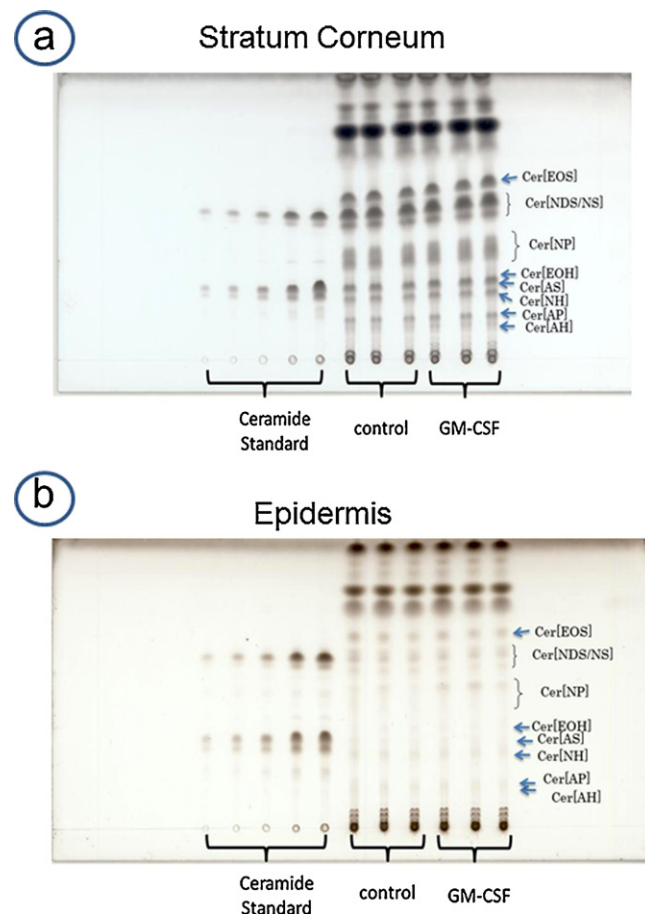


Fig. 2. TLC analysis for lipids from the stratum corneum and the epidermis. a: TLC analysis for lipids from the stratum corneum, b: TLC analysis for lipids for the epidermis. RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of GM-CSF at 10 nM and subjected to TLC analysis.

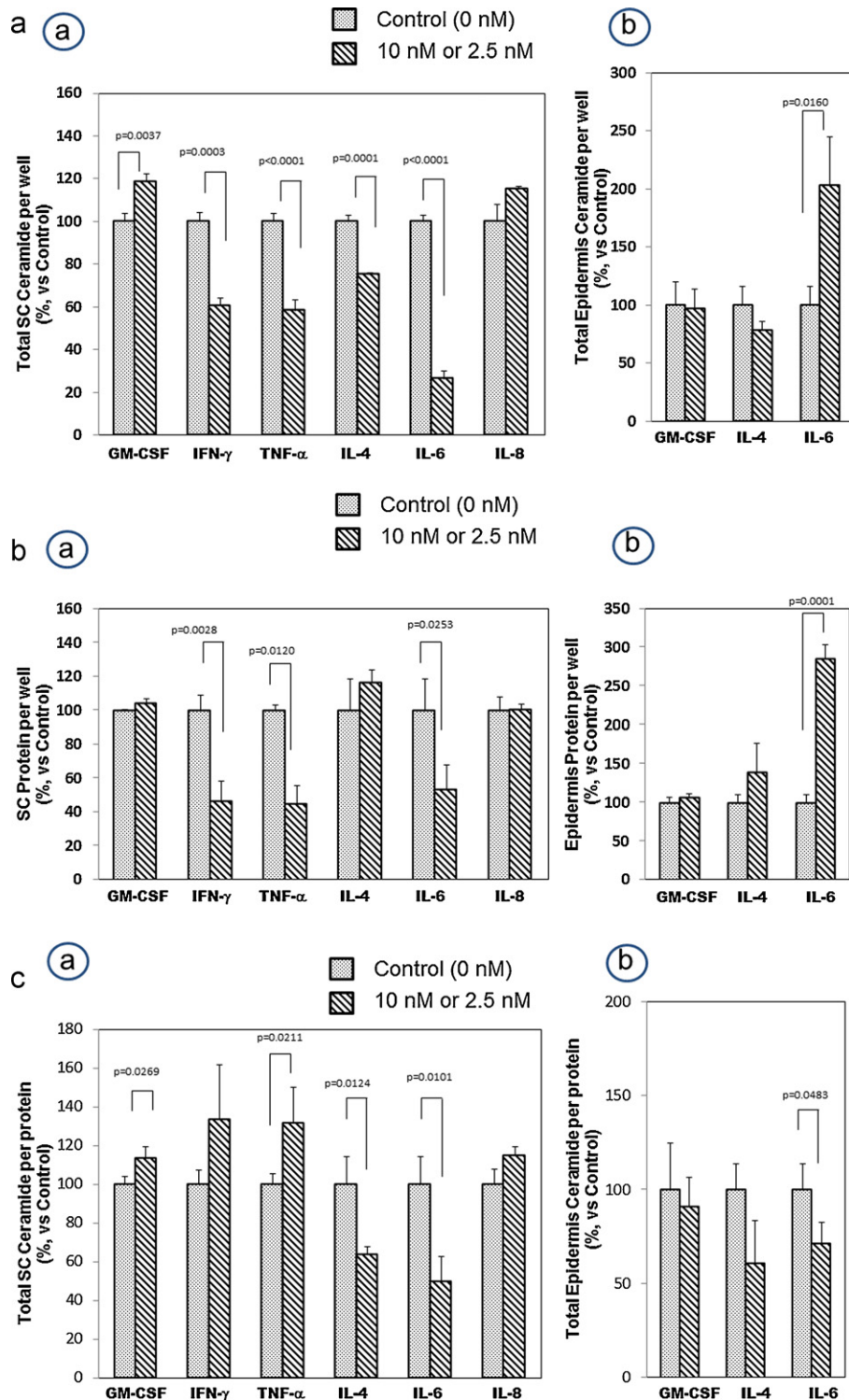


Fig. 3. Effects of cytokines on total ceramide levels in the SC and in the epidermis of RHEK models. a: total ceramide level/well. The actual SC ceramide values of control are 43.7 ± 1.6 $\mu\text{g}/\text{well}$ for GM-CSF experiment, 40.6 ± 1.7 $\mu\text{g}/\text{well}$ for IFN- γ , 28.4 ± 1.1 $\mu\text{g}/\text{well}$ for TNF- α , 48.4 ± 1.3 $\mu\text{g}/\text{well}$ for IL-4 and IL-6 and 58.1 ± 4.7 $\mu\text{g}/\text{well}$ for IL-8. The actual epidermis ceramide values of control are 3.7 ± 0.74 $\mu\text{g}/\text{well}$ for GM-CSF and 2.1 ± 0.3 $\mu\text{g}/\text{well}$ for IL-4 and IL-6. b: SC or epidermis protein level/well. The actual SC protein values of control are 264.8 ± 0.6 $\mu\text{g}/\text{well}$ for GM-CSF, 204.9 ± 17.6 $\mu\text{g}/\text{well}$ for IFN- γ , 205.3 ± 5.4 $\mu\text{g}/\text{well}$ for TNF- α , 241.0 ± 43.8 $\mu\text{g}/\text{well}$ for IL-4 and IL-6, and 267.8 ± 20.5 $\mu\text{g}/\text{well}$ for IL-8. The actual epidermis protein values of control are 65.1 ± 4.0 $\mu\text{g}/\text{well}$ for GM-CSF and 96.6 ± 8.7 $\mu\text{g}/\text{well}$ for IL-4 and IL-6. c: Total ceramide level/SC or epidermis protein. The actual SC ceramide/protein values of control are 165.0 ± 6.4 $\mu\text{g}/\text{mg}$ for GM-CSF, 198.9 ± 14.8 $\mu\text{g}/\text{mg}$ for IFN- γ , 139.3 ± 7.5 $\mu\text{g}/\text{mg}$ for TNF- α , 204.4 ± 28.7 $\mu\text{g}/\text{mg}$ for IL-4 and IL-6, and 217.3 ± 16.4 $\mu\text{g}/\text{mg}$ for IL-8. The actual epidermis ceramide/protein values of control are 57.2 ± 14.0 $\mu\text{g}/\text{mg}$ for GM-CSF and 22.1 ± 3.0 $\mu\text{g}/\text{mg}$ for IL-4 and IL-6. RHEK models were cultured at 37°C for 7 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN- γ) after which ceramide levels in the separated SC and epidermis were evaluated by TLC analysis. Values are means $\pm$ SD from 3 independent experiments. Only p values less than 0.05 which are considered statistically significant were displayed in each figure.

Table 1

Effects of cytokines on ceramide species levels in the SC of RHEK models.

		EOS	NDS/NS	NP	EOH	AS	NH	AP	AH
GM-CSF	0nM	29.5 ± 1.09	72.6 ± 4.40	31.1 ± 0.72	1.80 ± 0.45	14.04 ± 0.23	8.6 ± 0.42	5.1 ± 0.36	2.2 ± 0.31
	10nM	33.8 ± 3.12	85.0 ± 4.09 ⁺	32.4 ± 1.32	2.03 ± 0.11	15.74 ± 1.25	10.1 ± 0.65 ⁺	5.8 ± 0.47	2.6 ± 0.35
IFN-γ	0nM	28.1 ± 1.93	61.6 ± 4.09	49.9 ± 3.38	2.94 ± 0.41	23.29 ± 1.48	17.7 ± 2.35	9.5 ± 0.72	5.9 ± 1.44
	2.5 nM	29.0 ± 7.36	94.9 ± 21.25	62.1 ± 10.88	2.93 ± 0.70	29.84 ± 5.08	24.2 ± 5.95	15.2 ± 2.89 ⁺	7.7 ± 1.71
TNF-α	0nM	21.6 ± 1.69	44.8 ± 3.28	34.9 ± 2.18	2.38 ± 0.17	16.25 ± 0.49	10.6 ± 0.76	5.9 ± 0.48	2.8 ± 0.24
	10nM	22.0 ± 2.61	71.7 ± 10.68 ^{**}	37.2 ± 5.33	3.01 ± 0.73	24.15 ± 3.98	14.2 ± 2.10 ⁺	6.8 ± 1.00	4.1 ± 0.40 ^{**}
IL-4	0nM	38.3 ± 4.37	96.8 ± 14.00	39.0 ± 5.22	1.37 ± 0.44	13.79 ± 1.59	8.8 ± 1.59	4.6 ± 1.68	1.9 ± 0.53
	10nM	21.8 ± 1.06 ^{**}	48.9 ± 5.08 ^{**}	29.5 ± 2.08 ⁺	1.31 ± 0.14	11.08 ± 0.44 ⁺	8.3 ± 0.40	6.1 ± 0.45	3.1 ± 0.15 ⁺
IL-6	0nM	38.3 ± 4.37	96.8 ± 14.00	39.0 ± 5.22	1.37 ± 0.44	13.79 ± 1.59	8.8 ± 1.59	4.6 ± 1.68	1.9 ± 0.53
	10nM	12.4 ± 4.18 ^{**}	59.2 ± 13.10 ⁺	12.9 ± 4.03 ^{**}	0.51 ± 0.20	8.06 ± 1.52 ⁺	4.6 ± 1.62 ⁺	2.7 ± 0.94	2.1 ± 1.00
IL-8	0nM	27.1 ± 2.54	86.2 ± 9.55	51.0 ± 4.75	2.86 ± 0.49	21.31 ± 0.42	13.5 ± 1.80	9.8 ± 1.08	5.6 ± 0.90
	10nM	38.5 ± 1.61 ^{**}	97.2 ± 3.19	57.5 ± 2.75	2.68 ± 0.64	24.39 ± 1.51	15.7 ± 1.89	8.4 ± 0.36	5.0 ± 0.39

RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN-γ) after which ceramide species levels in the separated SC were evaluated by TLC analysis. Values represent SC ceramide species levels expressed as μg/mg protein and are means ± SD from 3 independent experiments.

⁺ $p < 0.05$.^{**} $p < 0.01$ vs 0nM.**Table 2**

Effects of cytokines on ceramide species levels in the epidermis of RHEK models.

		EOS	NDS/NS	NP	EOH	AS	NH	AP	AH
GM-CSF	0nM	18.30 ± 4.81	26.10 ± 7.05	4.06 ± 1.10	0.98 ± 0.01	1.49 ± 0.63	3.52 ± 0.53	1.67 ± 0.30	1.06 ± 0.66
	10nM	15.62 ± 1.62	20.42 ± 2.79	7.91 ± 3.52	1.25 ± 0.09	1.38 ± 0.11	2.99 ± 0.72	1.60 ± 0.43	0.83 ± 0.17
IL-4	0nM	16.47 ± 2.57	4.89 ± 0.08	0.06 ± 0.07	0.00 ± 0.00	0.10 ± 0.08	0.34 ± 0.17	0.06 ± 0.05	0.22 ± 0.09
	10nM	8.27 ± 2.03 ⁺	4.32 ± 2.42	0.11 ± 0.08	0.01 ± 0.00	0.13 ± 0.08	0.18 ± 0.11	0.07 ± 0.06	0.30 ± 0.35
IL-6	0nM	16.47 ± 2.57	4.89 ± 0.08	0.06 ± 0.07	0.00 ± 0.00	0.10 ± 0.08	0.34 ± 0.17	0.06 ± 0.05	0.22 ± 0.09
	10nM	5.30 ± 0.48 ^{**}	8.99 ± 1.75	0.51 ± 0.17 ⁺	0.01 ± 0.00	0.14 ± 0.05	0.63 ± 0.05 ⁺	0.05 ± 0.01	0.11 ± 0.02

RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of cytokines at 10 nM after which ceramide species levels in the separated epidermis were evaluated by TLC analysis. Values represent SC ceramide species levels expressed as μg/mg protein and are means ± SD from 3 independent experiments.

⁺ $p < 0.05$.^{**} $p < 0.01$ vs 0nM.

protein level/well (Fig. 3). Analysis of the composition of ceramide species demonstrated that among all ceramide species of the SC, only CER [AP] was significantly increased by IFN-γ treatment at 2.5 nM, while there were slight but not significant increases in other ceramide species (Table 1). There were significant increases in the expression levels of all genes encoding sphingolipid metabolic enzymes tested at day 4 of culture following treatment with IFN-γ at a concentration of 2.5 nM (Fig. 4). In contrast, the protein levels of aSMase (Fig. 5) and aCDase (Fig. 6) were slightly but not significantly down-regulated by the treatment with IFN-γ at 2.5 nM. The enzymatic activity of aSMase was significantly diminished at day 7 of culture, whereas that of BGCCase was significantly up-regulated (Fig. 7). Collectively, these findings indicate that IFN-γ slightly increases ceramide levels in the SC by up-regulating SPT-1, SPT-2 and aSMase at the transcriptional level and BGCCase at the both transcriptional and enzymatic activity levels.

3.3. Effects of TNF-α on SC ceramide levels and sphingolipid metabolic enzymes at the gene level

The addition of another TH1 cytokine, TNF-α, induced a marked hyper-keratinizing change in the epidermis (Fig. 1) and resulted in a significant increase of ceramide level (expressed as μg/mg protein in the SC) as a result of significant decreases in ceramide/well and in protein level/well (Fig. 3). Analysis of the composition of ceramide species demonstrated that among all ceramide species of the SC, CERs [NOS/NS], [NH] and [AH] were significantly increased by TNF-α treatment at 10 nM (Table 1). There were significant or slight increases in the expression of genes encoding all sphingolipid metabolic enzymes tested t day 4 of culture (Fig. 4). Collectively, these findings indicate that TNF-α increases ceramide levels in the SC by

up-regulating, SPT-1/2, aSMase and BGCCase at the transcriptional level.

3.4. Effects of IL-4 on SC or epidermis ceramide levels and sphingolipid metabolic enzymes at the gene, protein and enzymatic activity levels

In contrast to the TH1 cytokines, the addition of a Th2 cytokine, IL-4, induced exfoliative changes in the epidermis (Fig. 1) and resulted in a marked and significant decrease in ceramide levels (expressed as μg/mg protein and μg/well in the SC) (Fig. 3). In the epidermis, there was a slight but not significant decrease in epidermal ceramide levels expressed as μg/mg protein elicited by treatment with IL-4 (Fig. 3). Analysis of the composition of ceramide species demonstrated that among all ceramide species of the SC, CERs [EOS], [NDS/NS], [NP], [AS] and [AH] were significantly decreased by IL-4 treatment at 10 nM (Table 1), whereas there was a significant decrease of CER [EOS] in all ceramide species of the epidermis in IL-4 treated-RHEK models (Table 2). Whereas there was no change in the expression of genes encoding SPT-1 and aCDase at day 4 of culture, the expression of the genes encoding SPT-2, aSMase and BGCCase was significantly down-regulated by IL-4 (Fig. 4). In contrast to the decreased expression of the gene encoding aSMase, its protein expression was slightly but significantly increased by the treatment with IL-4 at 10 nM (Fig. 5), while there was a slight decrease in the protein level of aCDase (Fig. 6). The enzymatic activity of aSMase was significantly up-regulated by IL-4 at day 7 of culture, whereas that of BGCCase was significantly diminished (Fig. 7). Collectively, our results indicate that IL-4 reduces ceramide levels in the SC by down-regulating the expression of SPT-2 at the transcriptional level and BGCCase at the transcriptional and enzymatic activity levels, although the discrepancy between the gene and protein/activity of aSMase remains to be clarified.

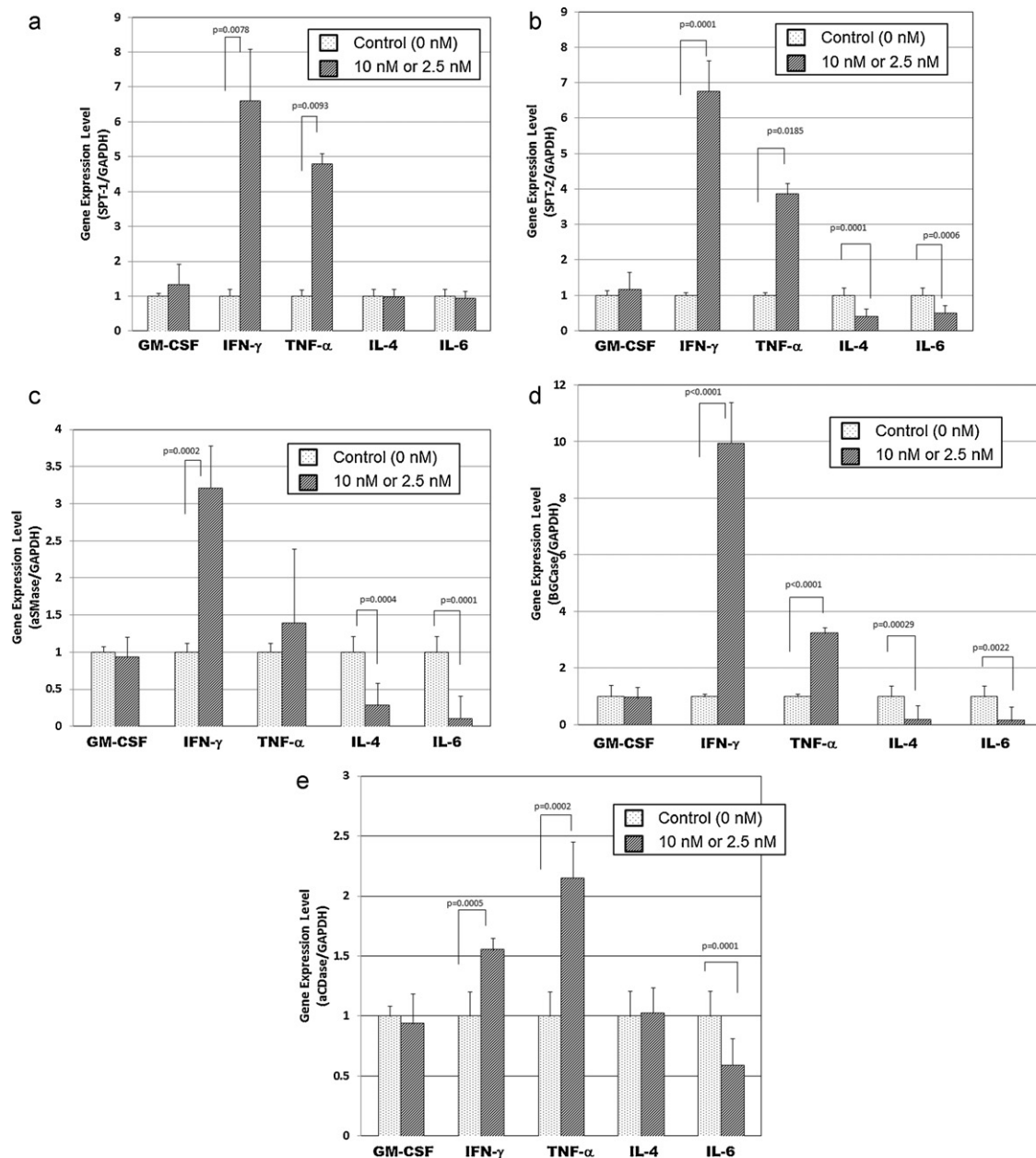


Fig. 4. Effect of cytokines on the expression of genes encoding SPT-1 (a), SPT-2 (b), aSMase (c), BGCase (d), and aCDase (e). RHEK models were cultured at 37 °C for 4 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN-γ) after which the gene expression levels of SPT-1 (a), SPT-2 (b), aSMase (c), BGCase (d), and aCDase (e) were evaluated in the whole epidermis including the SC by real-time RT-PCR analysis. Values are means $\pm$ SD from 6 independent experiments. Only *p* values less than 0.05 which are considered statistically significant were displayed in each figure.

3.5. Effects of IL-6 on SC ceramide levels and sphingolipid metabolic enzymes

The addition of IL-6 induced an abnormal keratinization pattern with residual nuclei in the SC (Fig. 1) and resulted in a marked and significant decrease in ceramide level/protein in the SC (Fig. 3). Similarly, there was a slight and significant decrease in epidermal ceramide levels expressed as $\mu\text{g}/\text{mg}$ protein in IL-6-treated RHEK models as a result of slight and marked increases in ceramide/well and in protein level/well, respectively (Fig. 3). Analysis of the composition of ceramide species demonstrated that among all ceramide species of the SC, CERs [EOS], [NDS/NP], [NP], [AS] and [NH] were significantly decreased by IL-6 treatment at 10 nM (Table 1), while there were a significant decrease of CER [EOS] and significant increases of CERs [NP] and [NH] in all ceramide species of the epidermis in IL-6-treated RHEK models (Table 2). The

expression of all genes encoding sphingolipid metabolic enzymes tested, except for SPT-1, was significantly down-regulated by IL-6 treatment (Fig. 4). Consistent with the gene expression analyses, the protein levels of aSMase (Fig. 5) and aCDase (Fig. 6) were significantly down-regulated by the treatment with IL-6 at 10 nM. The enzymatic activity of BGCase was significantly down-regulated at day 7 of culture, whereas that of aSMase was not altered by treatment with IL-6 at 10 nM (Fig. 7). Collectively, our results indicate that IL-6 reduces ceramide levels in the SC by down-regulating the expression of aSMase and BGCase as well as SPT-2.

3.6. Effects of IL-8 on SC ceramide levels

The addition of non-TH1/2 cytokine, IL-8, induced no degenerative change in the epidermis (Fig. 1) and resulted in no significant change of ceramide level (expressed as $\mu\text{g}/\text{mg}$ protein in the SC)

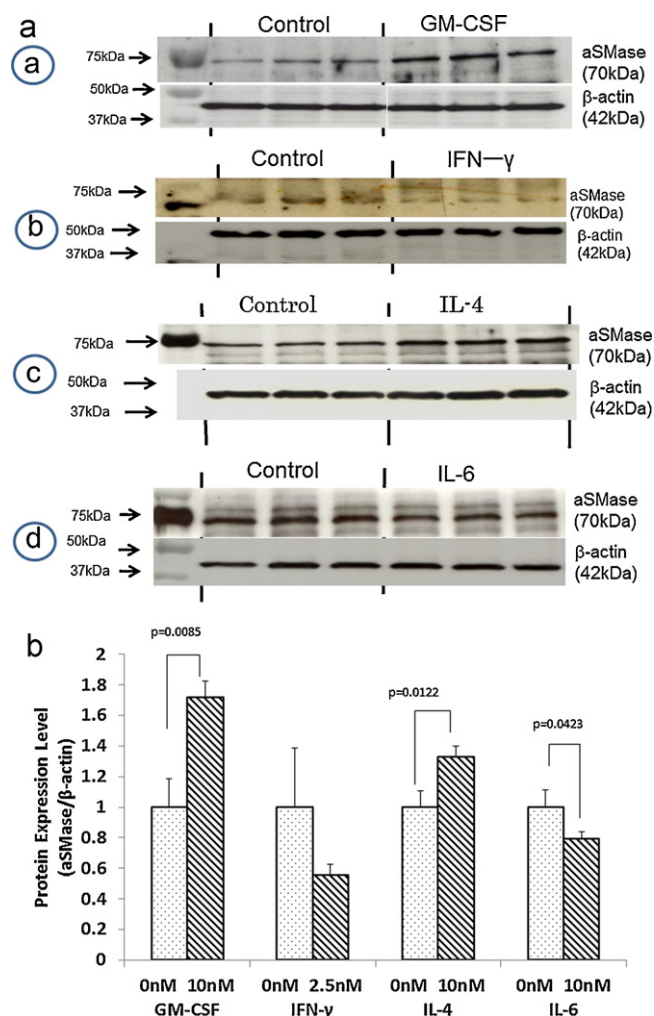


Fig. 5. Effect of cytokines on protein levels of aSMase. a: fluorography for western blotting; b: densitometric analysis. RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN-γ) after which the protein levels of aSMase were evaluated in the whole epidermis including the SC by Western blotting analysis. Values are means ± SD from 3 independent experiments. Only *p* values less than 0.05 which are considered statistically significant were displayed in each figure.

(Fig. 3). Analysis of the composition of ceramide species demonstrated that there was no significant difference in any ceramide species of the SC except for CER [EOS] between IL-8-treated and non-treated RHEK models (Table 1). Therefore, we did not measure the effects of IL-8 on the gene, protein and activity levels of several sphingolipid enzymes.

4. Discussion

The present study demonstrates the distinct regulatory effects of the balance of Th1/2 cytokine on ceramide levels in the SC. As summarized in Table 3, the addition of Th1 cytokines, such as GM-CSF, IFN-γ and TNF-α, distinctly increases the ceramide quantity expressed as μg/mg protein in the SC, whereas Th2 cytokines, such as IL-4 and IL-6, markedly decrease ceramide levels during 7 days of culture of the RHEK models. On the other hand, non-Th1/2 cytokines, such as IL-8, do not affect ceramide levels in the SC. Thus, the effects of the Th1/2 cytokine balance on ceramide levels in the SC show a distinct tendency towards a ceramide deficiency induced by Th2 cytokines and a ceramide increment induced by Th1 cytokines. Our mechanistic analyses indicate that a Th1 cytokine, GM-CSF, significantly increases the ceramide/protein

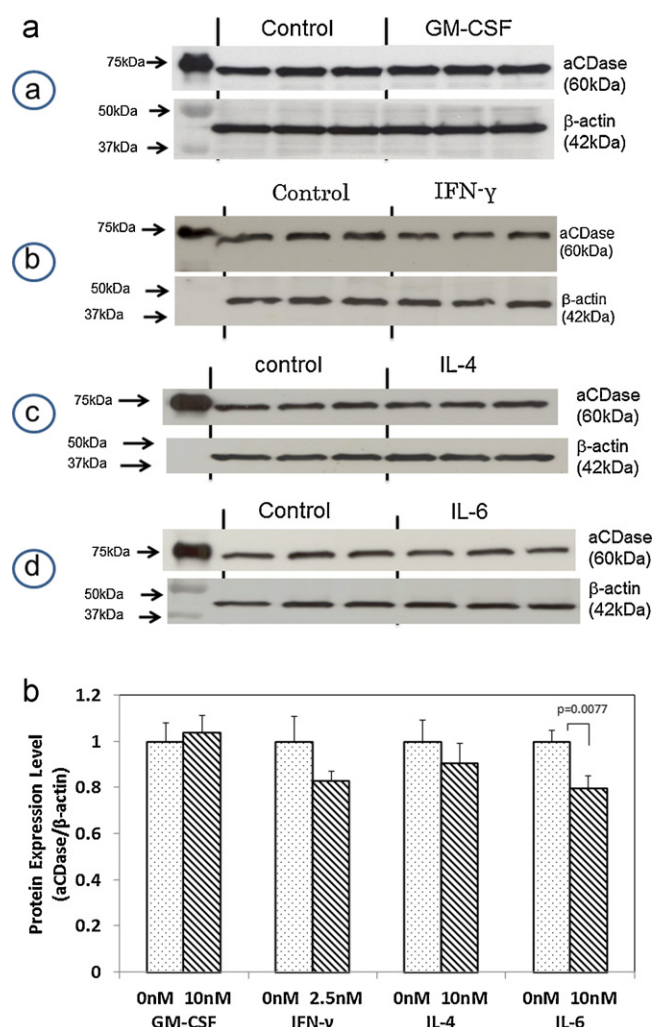


Fig. 6. Effect of cytokines on protein levels of aCDase. a: fluorography for western blotting; b: densitometric analysis. RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of cytokines at 10 nM or 2.5 nM (only for IFN-γ) after which the protein levels of aCDase were evaluated in the whole epidermis including the SC by Western blotting analysis. Values are means ± SD from 3 independent experiments. Only *p* values less than 0.05 which are considered statistically significant were displayed in each figure.

level in the SC by up-regulating both aSMase and BGCCase at the translational and post-translational levels. Similarly, another Th1 cytokine, IFN-γ, increases the ceramide/protein level in the SC by up-regulating SPT-1, SPT-2 and aSMase at the transcriptional level and BGCCase at the transcriptional and the enzymatic activity levels. Further, another Th1 cytokine, TNF-α, significantly increases ceramide/protein levels in the SC, accompanied by an up-regulated expression of SPT-1, SPT-2, BGCCase and aCDase at the transcriptional level. In contrast, a Th2 cytokine, IL-4, reduces ceramide/protein levels in the SC by markedly down-regulating the expression of SPT-2, aSMase and BGCCase at the transcriptional level and by suppressing the enzymatic activity of BGCCase, although a discrepancy between the gene and the protein/activity levels of aSMase remains to be clarified. Similarly, another Th2 cytokine, IL-6, reduces ceramide/protein levels in the SC by down-regulating the expression of SPT-2, aSMase and BGCCase at the transcriptional level and the expression of aSMase and BGCCase at the translational level, or by decreasing the enzymatic activity of BGCCase. The sum of these findings supports the hypothesis that Th1 cytokines stimulate ceramide production in the SC by up-regulating the expression of SPT-1, SPT-2, BGCCase and/or aSMase, whereas the Th2 cytokines reduce ceramide production in the SC

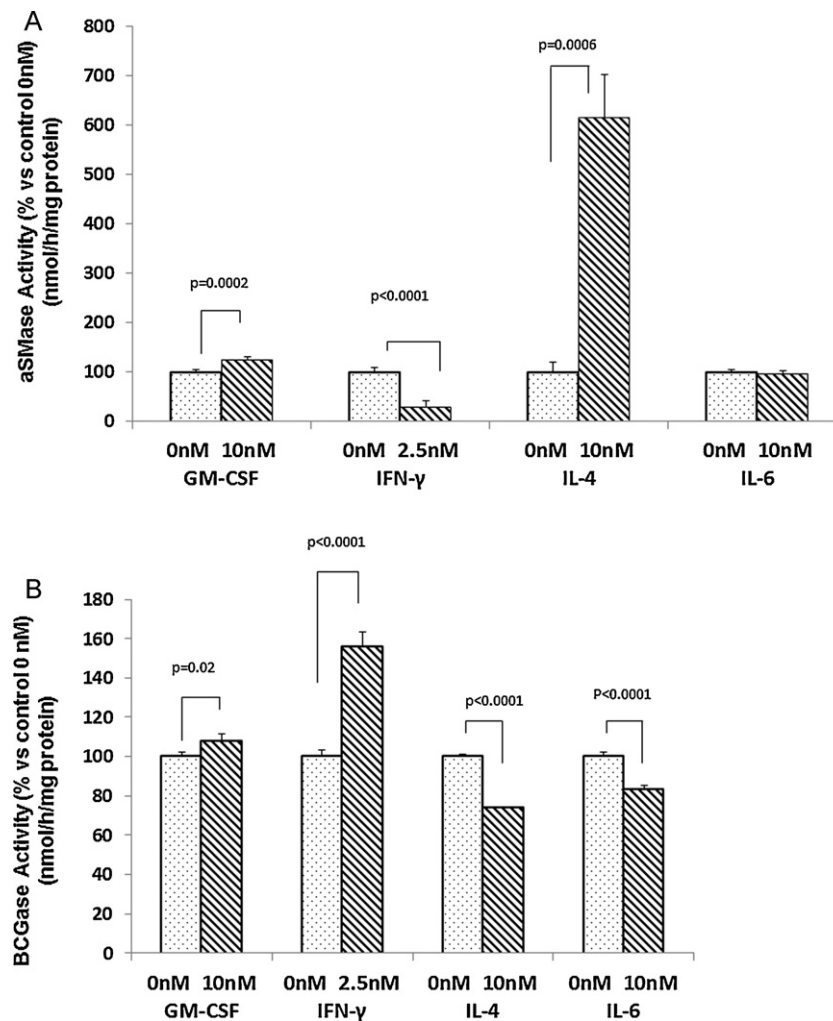


Fig. 7. Effect of cytokines on enzymatic activity of aSMase (A) and BGCase (B). A: aSMase activity, the actual SMase activity values of control are 3.22 ± 0.13 nmol/h/mg for GM-CSF and IL-6 experiments, 1.67 ± 0.15 nmol/h/mg for IFN-γ and 2.90 ± 0.57 nmol/h/mg for IL-4. B: BGCase activity. The actual BGCase activity values of control are 5.09 ± 0.13 nmol/h/mg for GM-CSF and IL-6, 0.50 ± 0.02 nmol/h/mg for IFN-γ and 0.45 ± 0.004 nmol/h/mg for IL-4. RHEK models were cultured at 37 °C for 7 days in DMEM in the absence or presence of IL-4 at 10 nM after which the activities of aSMase (A) and BGCase (B) were evaluated in the whole epidermis including the SC by enzymatic analysis. Values are means $\pm$ SD from 3 independent experiments. Only *p* values less than 0.05 which are considered statistically significant were displayed in each figure.

by down-regulating the expression of SPT-2, BGCase and/or aSMase. The effects on the expression of aCDase could not be reasonably accounted for the increasing or decreasing effects on ceramide/protein levels in the SC. These differential effects of

Th1/Th2 cytokines are in agreement with the general notion that the Th1 type of inflammation induced by UVB irradiation stimulates ceramide production in the SC in spite of the induced barrier disruption [29], and suggest the possibility that the Th2 type of inflammation evoked in AD skin results in a ceramide deficiency in the SC.

As for the mechanism(s) involved in the up- or down-regulation of ceramide synthesis in the SC, there are some complex cases where the stimulatory or suppressive effects of cytokines on the expression of genes encoding ceramide-metabolic enzymes are not paralleled by their effects at the translational and the enzymatic activity levels. This includes effects by GM-CSF, IFN-γ and IL-4. Although the reason for this discrepancy remains unclear, differences in sampling dates (days 4 and 7) between analyses for the gene and the protein/activity may be involved. Further, the up-regulated protein level without a corresponding increase in mRNA (as seen with aSMase following treatment with GM-CSF or IL-4) has been reported previously in other studies of cancer and innate immunity and is explained in terms of enhanced translation speed due to the up-regulation of translation control factors, such as initiation factor and/or elongation factor [30]. The most striking discrepancy occurs for the expression of aSMase following treatment with IL-4 where the protein and the enzymatic activity levels are significantly up-regulated despite the fact that aSMase

Table 3

Summary for the effects of cytokines on the total ceramide levels, the gene and protein expression of ceramide metabolic enzymes and their enzymatic activities.

	stratum corneum			epidermis			gene					protein		activity		
	ceramide/protein	ceramide/well	protein/well	ceramide/protein	ceramide/well	protein/well	SPT1	SPT2	aSMase	BGCase	aCDase	aSMase	aCDase	aSMase	BGCase	aCDase
GM-CSF	↑	↑↑	→	→	→	→	→	→	→	→	→	↑↑	→	↑↑	↑↑	↑
IFN-γ	↗	↓↓	↓↓	↘	↘	↘	↑↑	↑↑	↑↑	↑↑	↑↑	↘	↘	↘	↘	↑↑
TNF-α	↑	↓↓	↓	↘	↘	↘	↑↑	↑	↗	↑↑	↑↑	↘	↘	↘	↘	↘
IL-4	↓	↓↓	→	↘	↘	↘	→	↓↓	↓↓	↓↓	→	↑	↘	↑↑	↓↓	↓↓
IL-6	↓	↓↓	↓	↓	↑	↑↑	→	↓↓	↓↓	↓↓	↓↓	↓	↓↓	→	↓↓	↓↓
IL-8	↗	↗	→	↘	↘	↘	↘	↘	↘	↘	↘	↘	↘	↘	↘	↘

→: no change, ↗: a slight increase ($p > 0.05$), ↑: a moderate increase ($p < 0.05$), ↑↑: a marked increase ($p < 0.01$), ↘: a slight decrease ($p > 0.05$), ↓: a moderate decrease ($p < 0.05$), ↓↓: a marked decrease ($p < 0.01$).

gene expression is significantly down-regulated by IL-4. Although the discrepancy between the gene expression and the protein/activity levels for the mechanism(s) involved in the down-regulation of ceramide/protein level in the SC by IL-4 remains to be clarified, the reason why the distinctly increased levels of protein and enzymatic activity does not necessarily contribute to the increase in ceramide levels in the SC seems to be as follows: The inhibition or down-regulation of ceramide synthetic enzymes, such as SMase and BGDase, directly results in the diminished level of ceramides in the SC, leaving sphingomyelin and glucosylceramide as reaction residues in the SC, as seen in Niemann–Pick disease [31] and in Gaucher disease [32], respectively, or an *in vivo* inhibition study using a BGDase inhibitor [33] and in our study using human epidermal equivalents [23]. The stimulation of those activities does not necessarily reflect the increased level of ceramides in the SC because those enzyme activities occur at levels sufficient to completely degrade sphingomyelin and glucosylceramide without leaving their residues in the SC under the normal physiological conditions of keratinization. Thus, the increased activity of aSMase is not necessarily able to compensate for the decreased activity of BGDase as well as the possible decreased level of sphingomyelin due to the down-regulation of SPT-2 gene expression, so that despite the increased activity of aSMase, the IL-4 treatment may eventually diminish the ceramide level in the SC.

As for the effects of cytokines on ceramide species in the SC, while Th1 cytokines (GM-CSF, IFN- γ and TNF- α) increase mainly the level of Cer (NDS/NS), Th2 cytokines (IL-4 and IL-6) significantly diminish mainly the levels of Cer(EOS) and Cer(NDS/NS). These profiles are in good agreement with the fact that there is a significant decrease in the levels of Cer(EOS) and Cer(NDS/NS) in the lesional and non-lesional stratum corneum of AD skin compared with healthy controls [1]. Further, they also are consistent with the clinical observations that psoriatic skin has a marked up-regulation of secreted IL-6 [34,35], which is accompanied by a significant decrease in Cer(EOS) in the stratum corneum [36,37].

A previous study by Hatano et al. [21] on the effects on ceramide levels in the whole epidermis, including the SC (hereafter referred as the Epi-study), used TNF- α , IFN- γ and IL-4 as well as a combination of those 3 cytokines and demonstrated that TNF- α alone did not increase ceramide/protein levels in human epidermal sheets harvested from organ cultures of skin, which was accompanied by no significant effect on the gene expression levels of CDase, SMase and BGCase. In contrast, our study focusing on the ceramide/protein in the SC (hereafter referred as the SC-study) demonstrated that while TNF- α elicits a significant increase in ceramide/protein levels, there are significant increases in the expression of genes encoding SPT-1, SPT-2, BGCase and aCDase. The differential effects on gene expression levels of several sphingolipid metabolic enzymes between those two studies may reflect: (1) different durations of incubation with TNF- α (24 h vs 4 days) and/or (2) different concentrations of TNF- α (10 ng/ml vs 10 nM). On the other hand, while the Epi-study demonstrated that IFN- γ alone significantly increases the epidermal ceramide/protein level which is accompanied by a significant up-regulation of the gene expression level for BGCase but not for aCDase and aSMase, our SC study also reveals that IFN- γ induces a marked hyperkeratinizing change in the epidermis and results in a distinct increase of ceramide/protein level in the SC which is associated with the up-regulated gene expression of SPT-1, SPT-2, aSMase, BGCase, and aCDase. The difference in the gene expression of aSMase and aCDase may be due to the different duration of incubation (24 h vs 4 days) and the different concentrations of IFN- γ used (10 ng/ml vs 2.5 nM). In the Epi-study [21], although there is no data for the effect of IL-4 alone on the epidermal

ceramide/protein level, IL-4 significantly down-regulated the gene expression of BGCase but not aCDase or aSMase. In another Epi-study [22], while IL-4 alone significantly abolished the increased levels of epidermal ceramide/protein in acetone-treated skin equivalents, there was a significant down-regulation in the gene expression of BGCase and SMase but not of aCDase. Consistently, the same research group reported the suppressive effect of IL-4 on barrier recovery *in vivo* [38]. Our SC study demonstrates that IL-4 alone induces exfoliative changes in the epidermis and results in a marked decrease of ceramide/protein levels in the SC which is associated with a significant down-regulation in the expression of the genes encoding SPT-2, BGCase and aSMase but without any change in the gene expression of SPT-1 and aCDase, although aSMase is rather up-regulated at the translational and enzymatic activity levels. In addition, we demonstrate for the first time that another Th2 cytokine, IL-6, induces an abnormal keratinization pattern with residual nuclei in the SC and results in a marked and significant decrease of ceramide/protein level in the SC by down-regulating aSMase and BGCase as well as SPT-2 at the transcriptional and/or translational levels, which indicates suppressive effects that are similar to those elicited by IL-4. Further, we have also found for the first time that a Th1 cytokine, GM-CSF, distinctly increases ceramide/protein levels in the SC by up-regulating aSMase and BGCase at the translational and/or enzymatic activity levels, while IL-1 α [23] and IL-8 have no significant effect on ceramide/protein levels in the SC.

Among Th1-type cytokines, the ceramide up-regulation by IFN- γ and TNF- α seems to occur via mechanisms distinct from that elicited by GM-CSF. The increasing effect of GM-CSF is not accompanied by the up-regulated gene expression of aSMase and BGCase, or by the down-regulated gene expression of aCDase despite the fact that the enzymatic activities of aSMase and BGCase are significantly enhanced. In contrast, the increasing effect elicited by IFN- γ is distinctly associated with the up-regulated gene expression of SPT-1, SPT-2, aSMase and BGCase. These results suggest the possibility that GM-CSF triggers the activation process of aSMase and BGCase via the extrusion of lamellar bodies at the interface between the stratum granulosum and SC layers. Among Th2-type cytokines, there seems almost the same mechanism involved in the diminishing effects on ceramide/protein levels in the SC because the suppressive effects by IL-4 and IL-6 are distinctly associated with the down-regulated gene expression of aSMase and BGCase as well as SPT-2.

All hitherto reported evaluations related to the effects of Th1/Th2 cytokines on ceramide levels had been limited not to the SC, but to the whole epidermis [21]. However, although ceramide levels in the whole epidermis, including the SC, might roughly reflect levels in the SC because ceramides are predominantly located in abundance in the SC, one could not rule out the possibility that intracellular ceramide levels within living epidermal cells affects the total ceramide levels expressed/mg protein (due to an unbalanced change in ceramide and protein). This would result in errors in estimating ceramide levels in the SC which are essentially and precisely responsible for the barrier and water holding functions of the skin. In fact, in our other similar study using the same RHEK model [23], the comparison of ceramide/protein levels between the SC and epidermal cells through treatment with ceramide metabolic inhibitors revealed that there is no mutual correlation in ceramide/protein levels between the SC and the epidermal cells. This is quite reasonable when one considers the ceramide synthetic pathway in the SC where lamellar granules are secreted into intercellular spaces between SC cells during which the ceramide precursors, glucosylceramide and sphingomyelin, are hydrolyzed by BGCase and aSMase, respectively, indicating a major contribution of SC ceramide levels to such ceramide metabolic enzyme activity.

In conclusion, whereas the addition of Th1 cytokines, such as GM-CSF, IFN- γ and TNF- α results in a distinct increase in SC ceramides by up-regulating ceramide producing enzymes (especially sMase and/or BGCse), Th2 cytokines, such as IL-4 and IL-6, are associated with a marked decrease in ceramide levels in the SC by down-regulating SPT-2, sMase and BGCse. The sum of these findings suggests that the Th2 type of inflammation evoked in atopic dermatitis skin is an essential factor in down-regulating the levels of ceramides in the SC although the precise cellular mechanism(s) involved in cytokine-induced alteration of ceramide metabolism remains to be determined.

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