Interleukin-4 Suppresses the Enhancement of Ceramide Synthesis and Cutaneous Permeability Barrier Functions Induced by Tumor Necrosis Factor-α and interferon-γ in Human Epidermis

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Ceramide is an integral part of the extracellular lipid bilayer of the stratum corneum (SC) that forms the permeability barrier of the skin. The production of SC ceramides is catalyzed by sphingomyelinase (SMase) and glucocerebrosidase (GCase). Acid-ceramidase (acid-CDase) catalyzes the hydrolysis of ceramide in the SC. We examined the effects of T helper (Th)1 and Th2 cytokines on levels of transcripts of genes for acid-CDase, acid-SMase, and GCase, on levels of ceramide, and on the extent of transepidermal water loss (TEWL) in the human epidermis in an effort to determine whether these cytokines affect the permeability barrier functions. Levels of transcripts for acid-SMase and GCase and the amount of ceramide in human epidermal sheets were enhanced by tumor necrosis factor (TNF)-α and interferon (IFN)-γ and these effects were inhibited in the presence of interleukin (IL)-4. In epidermal keratinocytes cultured under submerged conditions, however, no similar inhibitory effects of IL-4 were observed. Consistent with these results, TEWL was suppressed by TNF-α and IFN-γ, and these effects were also inhibited by IL-4. The balance between Th1 and Th2 might affect the construction and/or the repair of the epidermal permeability barrier via regulation of the production of ceramide.

Key words: ceramide/skin/Th1 cytokines/Th2 cytokines/transepidermal water loss
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Ceramide is an integral part of the extracellular lipid bilayer of the stratum corneum (SC) that forms the permeability barrier of the skin (Elias and Feingold, 1992). In the scaly skin of patients with various inflammatory disorders, in particular, atopic dermatitis (AD), permeability barrier functions are disrupted, with increases in transepidermal water loss (TEWL) and decreases in levels of ceramide in the SC (Imokawa *et al*, 1991; Motta *et al*, 1994; Di Nardo *et al*, 1998; Tagami *et al*, 2001; Jensen *et al*, 2004), although it is unclear whether there are any abnormalities in the permeability barrier functions of the normal skin and non-active lesions of such patients (Imokawa *et al*, 1991; Di Nardo *et al*, 1998; Matsumoto *et al*, 1999; Sakurai *et al*, 2002).

It has been proposed that T helper (Th)2- and Th1-type cells play a key pathogenic role in the acute phase and the chronic phase, respectively, of AD (Grewe *et al*, 1998; Leung *et al*, 2004). The association between immunopathological features and cutaneous barrier functions, however, remains to be clarified. Relationships between the effects of barrier dysfunction and cutaneous inflammation have been discussed as follows. Di Nardo *et al* (1996) showed that cutaneous inflammatory reactions were enhanced in skin with low levels of ceramide. Abnormal dryness is known to be responsible for increased susceptibility to irritants and consequent impairment of barrier functions (Tupker *et al*, 1990). Nishijima *et al* (1997) showed that contact hypersensitivity was more severely augmented in acutely barrier-disrupted skin than in intact skin, not only as a result of increased permeability but also as a result of activation of immune reactivity. Kondo *et al* (1998) showed that, if percutaneous sensitization is performed in barrier-disrupted skin, a Th2-dominant reaction emerges in the afferent phase. By contrast, little information about the effects of cutaneous inflammation on barrier dysfunction has been reported. In particular, no studies of the relationship between the Th1/Th2 balance and the regulation of barrier functions have been reported to our knowledge.

In addition to being a major factor in both the permeability barrier and the maintenance of the water reservoir of the skin, ceramide, and/or its metabolites appear to be involved in the proliferation, differentiation, and apoptosis of epidermal keratinocytes (Wakita *et al*, 1994; Geilen *et al*, 1997). Moreover, Sallusto *et al* (1996) showed that ceramide inhibits the uptake and presentation of antigens by dendritic

Abbreviations: AD, atopic dermatitis; CDase, ceramidase; GCase, glucocerebrosidase; IFN, interferon; IL, interleukin; KGM, keratinocyte growth medium; LSE, living skin equivalent; NHEK, normal human epidermal keratinocytes; NHESheet, normal human epidermal sheet; PBS, phosphate-buffered saline; rh, recombinant human; SC, stratum corneum; SMase, sphingomyelinase; TEWL, transepidermal water loss; Th, T helper; TNF, tumor necrosis factor

cells in a murine model. It has also been proposed that ceramide modulates cytokine signals, for example, in the interleukin (IL)-1-mediated synthesis of prostaglandin and the interferon (IFN)-γ-induced expression of intercellular adhesion molecule-1 and of human leukocyte antigen-DR in keratinocytes (Ballou *et al*, 1992; Wakita *et al*, 1996; Kirtikara *et al*, 1998). Taken together, various observations suggest that the amount of ceramide in the epidermis might modulate immunological responses in the skin. Therefore, it seems to be appropriate to investigate the relationship between the production of ceramide and the balance between Th1 and Th2 cytokines as part of the ongoing efforts to elucidate the pathogenesis of various cutaneous disorders.

Skin ceramides are derived from newly synthesized compounds and from the hydrolysis of glucosylceramides and sphingomyelin (Holleran *et al*, 1993, 1994a; Jensen *et al*, 1999). SC ceramides 2 and 5 are produced by the hydrolysis of sphingomyelin by sphingomyelinase (SMase; Uchida *et al*, 2000) and the other groups of SC ceramides are produced by the degradation of glucosylceramide by glucocerebrosidase (GCase; Hamanaka *et al*, 2002). Several different forms of mammalian SMase have been identified (Schissel *et al*, 1996) and, in particular, acid-SMase is associated with the synthesis of the extracellular ceramide that acts as an intercellular lipid (Jensen *et al*, 1999). Both acid-SMase and GCase are essential for homeostasis of the permeability barrier (Holleran *et al*, 1994b; Jensen *et al*, 1999; Schmuth *et al*, 2000). Acid-ceramidase (acid-CDase), but not alkaline-CDase, catalyzes the hydrolysis of ceramide to sphingosine and fatty acid in the SC under physiological conditions (Yada *et al*, 1995; Arikawa *et al*, 2002; He *et al*, 2003). It is well known that inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1, and the prototypic Th1 cytokine IFN-γ stimulate the activity of SMase and suppress the activity of CDase. The resultant transient increase in levels of ceramide acts as a signal transducer (Hannun, 1994). Moreover, enhancement of SMase activity via the TNF-α receptor p55 is essential for repair of the permeability barrier (Jensen *et al*, 1999). The effects of these various agents on the levels of transcripts of these enzymes, however, have not been elucidated.

In this study, we examined the effects of Th1 (IFN-γ) and Th2 (IL-4) cytokines on levels of transcripts for acid-CDase, acid-SMase, and GCase, on the synthesis of ceramide in the epidermis, and on the TEWL in an attempt to determine whether the balance between Th1 and Th2 might affect the production of ceramide and the permeability barrier functions of the epidermis. Our results indicate that IL-4 might act as an inhibitor of the construction and/or the repair of the epidermal permeability barrier.

Results

Effects of Th1 and Th2 cytokines on the expression of mRNA for acid-CDase, acid-SMase, and GCase in cultured normal human epidermal keratinocytes (NHEK) As shown in Fig 1, levels of expression of mRNA for acid-CDase in cultured NHEK fell upon stimulation of cells by TNF-α and IFN-γ. Levels of mRNA for acid-SMase and GCase rose in response to TNF-α and IFN-γ. These effects of TNF-α and IFN-γ on the levels of transcripts for acid-CDase, acid-SMase, and GCase were unaffected by IL-4 (data not shown). Stimulation by IL-4 alone also did not affect the levels of transcripts for acid-CDase, acid-SMase, and GCase in cultured NHEK (data not shown). No effects of IL-4 could be detected in this system, even when the concentration of IL-4 was increased to 40 ng per mL.

Effects of Th1 and Th2 cytokines on the expression of mRNA for acid-CDase, acid-SMase, and GCase in normal human epidermal sheets (NHESheets) Similar to levels in cultured NHEK, levels of mRNA for acid-CDase, acid-SMase, and GCase fell and rose upon stimulation by

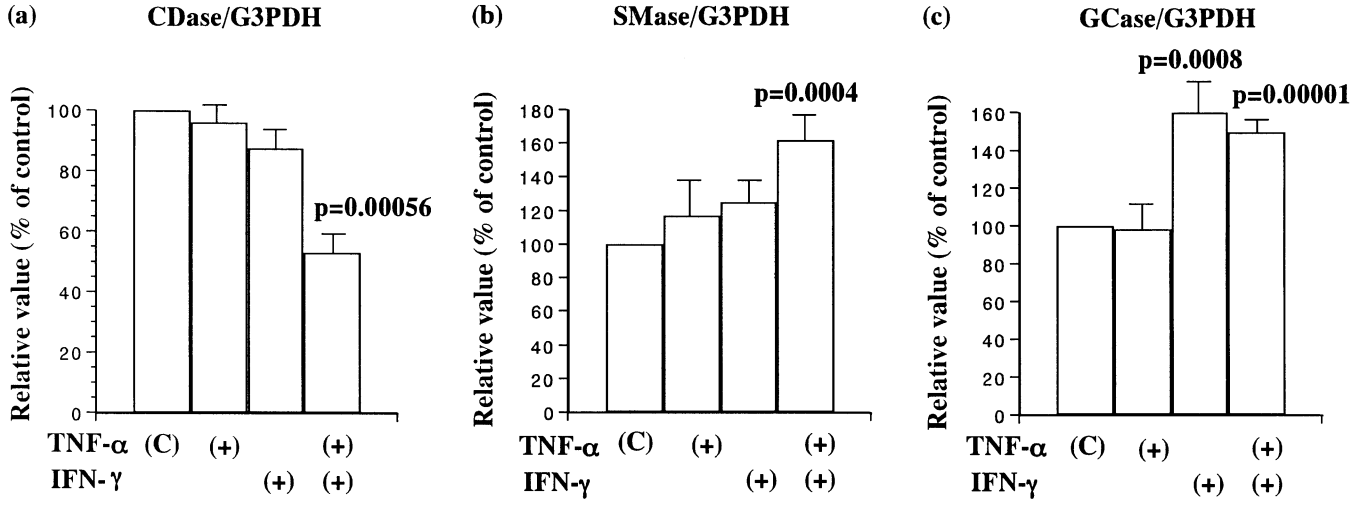

Figure 1
Effects of tumor necrosis factor (TNF)-α and interferon (IFN)-γ on levels of expression of mRNA for acid-ceramidase (acid-CDase), acid-sphingomyelinase (acid-SMase), and glucocerebrosidase (GCase) in cultured normal human keratinocytes. Levels of mRNA for acid-CDase, acid-SMase, and GCase in cultured normal human keratinocytes were examined by semiquantitative RT-PCR and normalized as described in Materials and Methods. The duration of incubation of cells in the presence of TNF-α (10 ng per mL) and/or IFN-γ (2 ng per mL) was 24 h. Data are expressed as percentages relative to levels of mRNA in non-stimulated controls (C). Columns and bars show means ± SEM; n = 10. p values refer to the significance of differences from the non-stimulated controls (C).

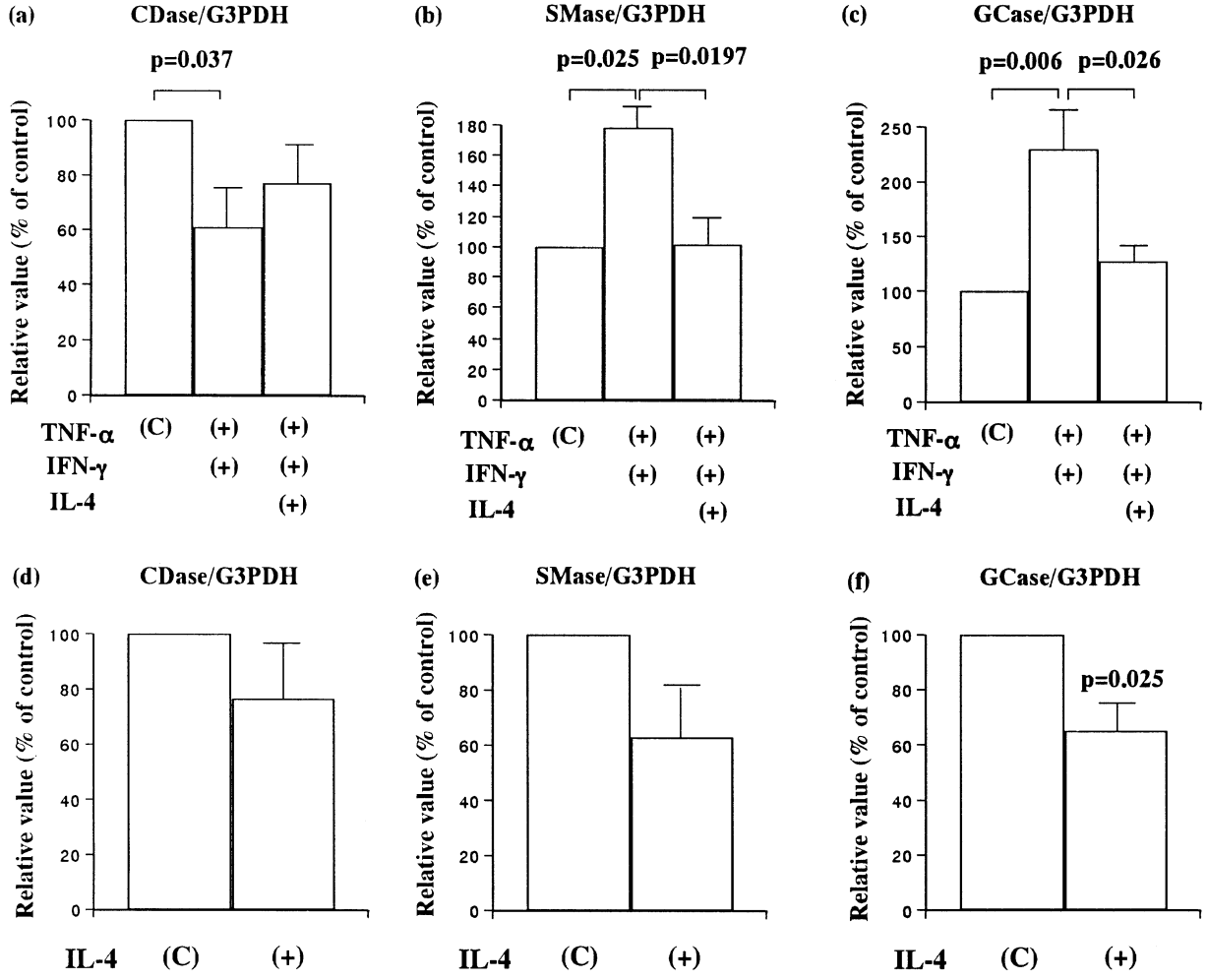

Figure 2
Effects of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-4 on levels of expression of mRNA for acid-ceramidase (acid-CDase), acid-sphingomyelinase (acid-SMase), and glucocerebrosidase (GCase) in normal human epidermal sheets. Levels of mRNA for acid-CDase, acid-SMase, and GCase in cultured normal human epidermal sheet were examined by semiquantitative RT-PCR and normalized as described in Materials and Methods. The duration of incubation in the presence of TNF-α (10 ng per mL), IFN-γ (2 ng per mL), and/or IL-4 (20 ng per mL) was 24 h. Data are expressed as percentages relative to levels of mRNA in non-stimulated controls (C). Columns and bars show means ± SEM. For (*a*)–(*c*), n = 4; for (*d*)–(*f*), n = 3.

TNF-α and IFN-γ, respectively, as shown in Fig 2. But in NHESheets, as distinct from cultured NHEK, the enhancing effects of TNF-α and IFN-γ on the expression of mRNA for acid-SMase and GCase were suppressed by IL-4. By contrast, IL-4 did not affect the suppressive effects of TNF-α and IFN-γ on the expression of the mRNA for acid-CDase. Stimulation by IL-4 alone depressed the level of transcripts for GCase in NHESheets and the effect was statistically significant. Levels of mRNA for acid-SMase also tended to be suppressed by IL-4 but the suppression was not statistically significant ($p = 0.106$). Stimulation by IL-4 alone did not affect levels of transcripts for acid-CDase.

Effects of Th1 and Th2 cytokines on total amounts of ceramide in NHESheets harvested from organ cultures of skin and living skin equivalent (LSE) Levels of ceramide in NHESheets obtained from normal human skin barely changed in the absence of cytokine stimulation (data not shown). As shown in Fig 3, however, they rose upon stimulation by TNF-α and IFN-γ. The effects of TNF-α and IFN-γ were suppressed by IL-4. The suppressive effects of IL-4 were also evident in NHESheets obtained from LSE. The amounts of ceramide in NHESheets were unaffected by IL-4 alone (data not shown), and the amounts of ceramide in cultured NHEK were unaffected by stimulation by TNF-α, IFN-γ, or IL-4 (data not shown).

Effects of Th1 and Th2 cytokines on TEWL in LSE The extent of TEWL in LSE was reduced upon stimulation by TNF-α and IFN-γ, and the effects of TNF-α and IFN-γ were suppressed by IL-4, as shown in Fig 4.

Discussion

Stimulation by TNF-α and IFN-γ depressed levels of the transcripts for acid-CDase and enhanced those for acid-SMase and GCase in cultured NHEK and NHESheets. Moreover, the amount of ceramide in NHESheets obtained from skin explants increased upon stimulation by TNF-α and IFN-γ, as compared with that obtained from non-stimulated skin explants. These results suggest that Th1-predominant inflammation might be one of the factors that

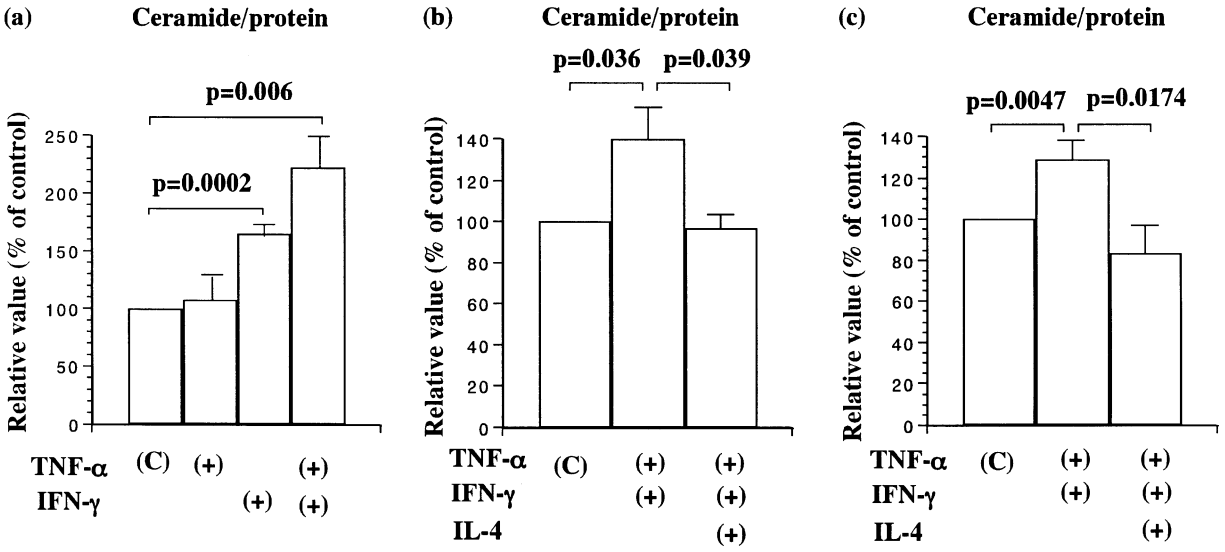

Figure 3
Effects of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-4 on total amounts of ceramide in normal human epidermal sheets (NHESheets) harvested from organ cultures of skin and from living skin equivalents (LSE). Total amounts of ceramide in NHESheets (*a*, *b*) harvested from organ cultures of skin and from LSE (*c*) were examined as described in Materials and Methods. The duration of incubation in the presence of TNF-α (10 ng per mL), IFN-γ (2 ng per mL), and/or IL-4 (20 ng per mL) was 72 h. Data are expressed as percentages relative to the total amounts of ceramide in non-stimulated controls (C). Columns and bars show means ± SEM. (*a*) NHESheets, n = 4; (*b*) NHESheets, n = 6; (*c*) LSE, n = 6.

promote the production of ceramide in the epidermis, although it remains to be determined whether the alterations in levels of transcripts for these enzymes are correlated with changes in the production of ceramide. In addition, other ceramide-generating enzymes, such as serine palmitoyl transferase, might affect the increase in the production of ceramide (Holleran *et al*, 1991). The effects of TNF-α and IFN-γ, however, seem reasonable with respect to homeostasis of the permeability barrier, reflecting the enhanced expression of GCase and acid-SMase after acute disruption of this barrier (Holleran *et al*, 1994a, b; Jensen *et al*, 1999; Schmuth *et al*, 2000). The involvement of Th1 cytokines in the pathogenesis of AD (Grewe *et al*, 1998; Leung *et al*, 2004) suggests that our results support previous reports of increases in the amount of acid-SMase (Kusuda *et al*, 1998) and decreases in the activity of acid-CDase (Arikawa *et al*, 2002) in atopic lesional skin.

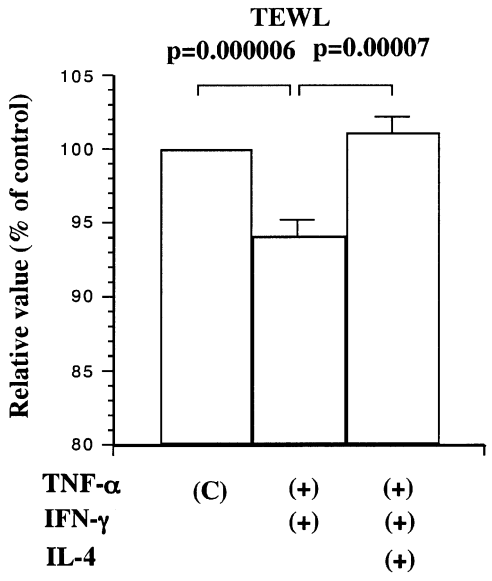

Figure 4
Effects of T helper (Th)1 and Th2 cytokines on transepidermal water loss (TEWL) in living skin equivalents. The extent of TEWL in living skin equivalents (LSE) was examined as described in Materials and Methods. The duration of incubation in the presence of tumor necrosis factor (TNF)-α (10 ng per mL), interferon (IFN)-γ (2 ng per mL), and/or interferon (IL)-4 (40 ng per mL) was 72 h. Data are expressed as percentages relative to the TEWL in non-stimulated controls (C). Columns and bars show means ± SEM; n = 11.

It was interesting to note that IL-4, a prototypic Th2 cytokine, suppressed the enhancing effects of TNF-α and IFN-γ on levels of transcripts for acid-SMase and GCase and on the production of ceramide in epidermal sheets. The mechanism of this effect of IL-4 remains unknown but our results suggest that IL-4 might act as a suppressor of barrier homeostasis and this suppressive effect of IL-4 might be related to the decrease in ceramide in the SC in atopic lesions. The impaired SMase activity in AD that was demonstrated recently by Jensen *et al* (2004) supports this hypothesis. It is unclear, however, whether the suppressive effect of IL-4 on ceramide production, as demonstrated in this study, is involved in the decrease in levels of ceramide observed in the SC in cases of AD because (i) we did not examine the effect of IL-4 on the activity of sphingomyelin acylase, which appears to be a key enzyme in the decrease in levels of ceramide in AD (Murata *et al*, 1996), and (ii) AD is not always in the Th2-dominant state, as mentioned in the Introduction.

It is unclear why the effects of IL-4 on levels of transcripts of acid-SMase and GCase differed between cultured NHEK and NHESheets. The difference might be related to differentiation of keratinocytes and the differences in cellular fatty acids (Terashi *et al*, 2000), which affect the regulation of transcription (Jump, 2004).

The effects of TNF-α, IFN-γ, and IL-4 on the amount of ceramide in epidermal sheets were reproduced in

experiments with LSE. This observation suggests that the targets of the effects of TNF-α, IFN-γ, and IL-4 in the regulation of ceramide levels in epidermal sheets were keratinocytes because the epidermis of LSE consists of human keratinocytes.

A particularly interesting result of this study was the decrease in TEWL in LSE upon stimulation by TNF-α and IFN-γ and the increase upon additional stimulation by IL-4. Since ceramide is one of the important components of the permeability barrier of the skin, as mentioned above, the results relating to TEWL are consistent with our results for levels of ceramide and the two sets of results reinforce each other. Moreover, this analysis of TEWL might explain the relationship between the increased TEWL and allergic inflammation in atopic skin, even though we did not examine the actual effects of inflammation. This study seems, at least, to indicate that a Th2 cytokine might act as an inhibitory factor during the repair of a cutaneous barrier that has been damaged by cutaneous inflammation. This hypothesis might be compatible with the previous demonstration that disturbance of epidermal barrier functions can be induced by patch test reactions to aeroallergens, which resemble lesional atopic eczema macroscopically, microscopically, and immunopathologically, but not by patch test reactions to contact allergens (Gfesser *et al*, 1996). But the effects of Th1 and Th2 cytokines on other important factors in the permeability barrier, for example, those involved in the secretion of the contents of lamellar granules into the intercellular domain, the formation of intercellular multilamellar lipid sheets (Schwarzendruber *et al*, 1987; Fartasch *et al*, 1992; Matsuki *et al*, 1998), and other epidermal lipids that are important for permeability barrier function, such as cholesterol and/or fatty acids (Wertz, 2000), remain unknown.

In conclusion, this study suggests that Th1 and Th2 cytokines might act in different ways to regulate the production of ceramide that leads to barrier repair and/or homeostasis. Further studies to determine directly the effects of Th1 and Th2 cytokines on barrier repair and/or homeostasis are now necessary.

Materials and Methods

Culture of NHEK Normal human skin was obtained from fresh surgically resected specimens in compliance with the Declaration of Helsinki guidelines and after informed consent had been obtained from each patient. Samples of skin were incubated with phosphate-buffered saline (PBS) that contained 0.05% trypsin (DIFCO, Detroit, Michigan) and 0.02% EDTA overnight at room temperature. After treatment with 0.05% trypsin inhibitor (Sigma, St Louis, Missouri) for a few seconds, the epidermis was peeled away from the dermis. A suspension of single cells was obtained by vigorous pipetting. Cells were washed and cultured in keratinocyte growth medium (KGM), which consisted of MCDB 153 medium (Sigma), 0.09 mM $CaCl_2$, 0.4 mg per mL hydrocortisone (Sigma), 5 mg per mL insulin (Eli Lilly Japan K.K., Kobe, Japan), 5 ng per mL recombinant human (rh) epidermal growth factor (R&D Systems, Minneapolis, Minnesota), and 50 mg per mL bovine pituitary extract (Biomedical Technologies Inc. Stoughton, MA, USA), at 37°C in a humidified atmosphere of 5% CO2 in air. Third- to fifth-passage cells were used for all experiments when they had reached 60%–70% confluence.

Culture of epidermal sheets Samples of normal human skin were cut into disks (6 mm in diameter) with a punch. Epidermal sheets were separated from each disk by treatment with 2000 IU per mL dispase (Godo Shusei, Tokyo, Japan) for approximately 1 h at 37°C. Epidermal sheets were floated in modified KGM that consisted of MCDB 153 medium, 0.4 mg per mL hydrocortisone, 5 mg per mL insulin, and an elevated concentration of $CaCl_2$ (1.8 mM) to prevent tissue degeneration (Tavakkol *et al*, 1999), at 37°C in a humidified atmosphere of 5% CO_2 in air.

Organ culture of skin Disks of human skins, prepared as described above, were cultured in modified KGM at 37°C in a humidified atmosphere of 5% CO_2 in air. LSE (TESTSKIN-LSE high, Toyobo, Osaka, Japan), which is a model of reconstructed skin, was incubated in the same modified medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The surface of the epidermis was kept under air-exposed conditions. The survival of organ cultures was confirmed by a standard MTT assay, according to the manufacturer's protocol (Toyobo; data not shown).

Stimulation with cytokines NHEK, epidermal sheets, skin explants, and LSE were stimulated with 10 ng per mL rh TNF-α (Invitrogen, Carlsbad, California), 2 ng per mL rh IFN-γ (Gibco BRL, Gaithersburg, Maryland), and 20 or 40 ng per mL rh IL-4 (R&D Systems). Incubation was continued for 24 h for NHEK and epidermal sheets and for 72 h for skin explants and LSE.

Semiquantitative RT-PCR We examined the expression of mRNA for acid-CDase, acid-SMase, and GCase in NHEK and epidermal sheets after stimulation by cytokines by semiquantitative RT-PCR, which was performed as described previously (Katagiri *et al*, 1997). Fifty nanograms of RNA were reverse-transcribed using random hexamers (Takara Shuzo, Shiga, Japan) and MMLV reverse transcriptase (Gibco BRL). The resultant cDNA was amplified by PCR in the presence of 1 μCi 32P-dCTP. We performed PCR for the appropriate number of cycles (1 min at 94°C; then 1 min at 57°C for acid-CDase, at 58°C for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), or at 60°C for acid-SMase and GCase; and 1 min at 72°C) within the linear ranges of amplification as follows: 20 cycles for G3PDH; 22 cycles for acid-CDase; and 24 cycles for acid-SMase and GCase. Aliquots (1 μL) of each sample after amplification were analyzed by electrophoresis on a 5% acrylamide/Tris-borate EDTA gel and products were visualized by autoradiography. DNA was quantified by phosphor image analysis (BAS2000; Fuji Medical Systems, Stanford, Connecticut). Levels of mRNA for acid-CDase, acid-SMase, and GCase were normalized by reference to levels of mRNA for G3PDH. The oligonucleotide primers used for PCR were based on published sequences of transcripts. The primers for amplification of cDNA for human G3PDH were 5′-CCCATCACCATCTTCCAG-3′ for the upstream primer and 5′-CCTG CTTCA CCACCACCTTCT-3′ for the downstream primer. For acid-CDase, we used 5′-CCTTCTTCCTTGATGATCGC-3′ as the upstream primer and 5′-GTGGAGTTCACCA TGGTTCG-3′ as the downstream primer; for acid-SMase, we used 5′-TGGCTCTATGA AGCGATGG-3′ as the upstream primer and 5′-AGGCCGATGTAGGTAGTTGC-3′ as the downstream primer; and for GCase we used 5′-TGGCATTGCTGTACATTGG-3′ as the upstream primer and 5′-CGTTCT TCTGACTGGCAACC-3′ as the downstream primer. We identified the products of PCR by the following procedures. Each reaction mixture was subjected to electrophoresis on an agarose gel and then bands of amplified fragments were extracted and digested with an appropriate restriction endonuclease. The sizes of the products of PCR and of the fragments were compared with the expected sizes by reference to mobilities of molecular size markers (100-bp ladder; Gibco BRL).

Analysis of lipids After stimulation by cytokines, epidermal sheets were collected from skin explants and LSE by treatment with dispase. Lipids were extracted as described previously (Terashi *et al*, 2000). The lipids in epidermal sheets were extract-

ed with a mixture (1:2:1.5, vol/vol) of methanol, chloroform, and 0.1 M KCl in 50% methanol, and the organic phase was re-extracted with 2.5 vol of 0.1 M KCl in 50% methanol. Precipitated proteins were quantitated by a modified version of Lowry's assay with bovine serum albumin as the standard protein. The extracted fraction was suspended in a mixture (1:1, vol/vol) of chloroform and methanol after evaporation under a stream of nitrogen gas and was applied to a thin-layer chromatography plate, as described by Imokawa *et al* (1991). The plate was developed with a mixture (190:9:1, vol/vol) of chloroform, methanol, and acetic acid. After solvent development, the chromatogram was air-dried, sprayed with an aqueous solution of 10% $CuSO_3$ and 8% H_3PO_4, and charred at 180°C. The charred lipids were quantitated by photodensitometry. Ceramides were quantitated by reference to appropriate commercial standards as follows: ceramide type III and ceramide type IV (Sigma) were used as standards for ceramides 1 and 2 and for ceramides 3, 4, 5, and 6, respectively. The amounts of ceramide were normalized by reference to amounts of protein from the epidermal sheets.

Measurement of TEWL in LSE After incubation as mentioned above, LSE was placed on a sponge that had been wetted with PBS, with the dermal side facing the sponge. Approximately 30 min later, we measured TEWL with a "Tewameter" (TM300; Integral, Tokyo, Japan) at room temperature and ambient humidity.

Statistical analysis Results were compared, and the statistical significance of differences was evaluated by Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

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