ORIGINAL ARTICLE

See related commentary on pg 597

Interferon-γ Decreases Ceramides with Long-Chain Fatty Acids: Possible Involvement in Atopic Dermatitis and Psoriasis

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Ceramide (CER) with long-chain fatty acids (FAs) in the human stratum corneum (SC) is important for the skin barrier functions. Changes in the CER profile have been associated with abnormal permeability of dermatoses such as atopic dermatitis (AD) and psoriasis. In addition, interferon-γ (IFN-γ) has been known to be abundant in both AD and psoriatic skin lesions. In this study, we aimed to identify the mechanism underlying the alteration of FA chain length of CERs in these diseases. Mass spectrometry analysis of CERs in the SC showed that the proportion of CERs with long-chain FAs was significantly lower in AD and psoriasis patients than in healthy controls, and this reduction was more pronounced in psoriasis than in AD. Using cultured human keratinocytes and epidermal sheets, we found that only IFN-γ among various cytokines decreased the mRNA expression of elongase of long-chain fatty acids (ELOVL) and ceramide synthase (CerS), enzymes involved in FA chain elongation. Furthermore, quantitative analysis showed that IFN-γ decreased the levels of CERs with long-chain FAs. These results suggest that IFN-γ decreases CERs with long-chain FAs through the downregulation of ELOVL and CerS and that this mechanism may be involved in the CER profile alteration observed in psoriasis and AD.

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INTRODUCTION

In the human stratum corneum (SC), ceramides (CERs) are responsible for maintaining a hydrophobic environment, and they have an important role in the development and maintenance of the water permeability barrier of the skin (Choi and Maibach, 2005; Feingold, 2007). Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by abnormality in SC permeability. The changes in the CER profile of AD patients have been explored extensively, and the characteristic abnormality in SC permeability has been associated with decreased CER levels (Imokawa *et al.*, 1991; Di Nardo *et al.*, 1998; Choi and Maibach, 2005). Psoriasis is an immune-mediated inflammatory and proliferative disorder of the skin that causes hyperkeratotic skin lesions. Changes in the CER profile of psoriasis patients have also been associated with impaired barrier functions (Motta *et al.*, 1993, 1994); however, the fatty acid (FA) profile of CERs in psoriasis is not well studied.

A recent study revealed that there are 342 CER species, which can be divided into 11 classes (Masukawa *et al.*, 2008). Further, improved SC barrier function strongly correlates with the average FA chain length of CERs (Ishikawa *et al.*, 2010; Joo *et al.*, 2010; Janůšová *et al.*, 2011). Thus, both the amount and the FA profiles of CERs are critical for SC barrier function. Therefore, in this study, we analyzed the CER profiles of SC in AD and psoriasis patients by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). To elucidate the mechanisms underlying the alteration of FA length in CERs, we focused on two enzymes: elongase of long-chain fatty acids (ELOVL) and CER synthase (CerS), which are involved in the elongation of FAs of CERs (Mizutani *et al.*, 2009; Guillou *et al.*, 2010). Both AD and psoriasis are associated with immunological abnormalities, including dysregulation of T helper (Th) type 1 and type 2 (Th1/Th2) and Th1/Th17 cells, respectively (Del Prete, 1992; Nestle *et al.*, 2009). In addition, interferon-γ (IFN-γ), a Th1 cytokine, has been shown to be highly expressed in the skin lesions of both diseases (Barker *et al.*, 1991; Grewe *et al.*, 1994, 1998; Szabo *et al.*, 1998; Lowes *et al.*, 2008; Bieber, 2010). Therefore, we examined the effects of various cytokines on the mRNA expression levels of ELOVL and CerS using normal human epidermal keratinocytes (NHEKs) and reconstructed three-dimensional epidermis.

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Abbreviations: AD, atopic dermatitis; CER, ceramide; CerS, ceramide synthase; ELOVL, elongase of long-chain fatty acids; FA, fatty acid; IFN, interferon; LCMS-IT-TOF, ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NHEK, normal human epidermal keratinocyte; SC, stratum corneum

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RESULTS

Decreased molecular size of CERs in tape-stripped SC of AD and psoriasis patients by MALDI-TOF-MS analysis

Sixty-three peaks above background noise, which consisted of 8 CER classes and covered 297 CER species out of 342 species, were detected between *m/z* values of 608 and 820 (Supplementary Figure S1 online). Three classes of omega-hydroxy CERs, i.e, CER[EOS], CER[EOH], and CER[EOP] were not detected. Distributions of total carbon numbers of CERs in AD and psoriasis patients were analyzed. The proportion of CER[NH] with total carbon number between 40 and 43 (C_{40}–C_{43} CER[NH]) was higher, whereas that of C_{47}–C_{50} CER[NH] was lower in both AD and psoriasis patients than in healthy controls (Figure 1a and c). The proportion of C_{44}–C_{46} CER[NH] decreased only in psoriasis (Figure 1b). Similar changes in CER[ADS]/[NP] and CER[AP] levels were observed in psoriasis but not in AD; the proportion of C_{38}–C_{43} CER[ADS]/[NP] and C_{38}–C_{42} CER[AP] increased, whereas that of C_{49}–C_{52} CER[ADS]/[NP] and C_{45}–C_{48} CER[AP] decreased. Significant difference in the proportion of C_{44}–C_{48} CER[ADS]/[NP] was also observed between psoriasis patients and controls (Figure 1d–i). No obvious difference was observed in the proportion profiles of CER[AH]/[NS] and CER[NDS]/[AS] among psoriasis, AD, and control (data not shown).

IFN-γ reduced mRNA levels of ELOVL and CerS in NHEKs

The effects of cytokines involved in the pathophysiology of these diseases on ELOVL and CerS mRNA expression were examined in cultured NHEKs. The NHEKs expressed ELOVL1, ELOVL4, ELOVL5, ELOVL6, ELOVL7, CerS2, CerS3, CerS5, and CerS6, whereas the expression levels of ELOVL2, ELOVL3, CerS1, and CerS4 were minimal (Supplementary Figure S2 online). When NHEKs were stimulated with interleukin (IL)-1, IL-4, IL-5, IL-8, IL-17, IL-18, IFN-γ, TNF-α (tumor necrosis factor-α), or GM-CSF (granulocyte macrophage colony-stimulating factor), only IFN-γ reduced the expression levels of ELOVL4, ELOVL5, CerS3, and CerS6 by 84, 39, 52, and 62%, respectively (Figure 2). Expression levels of other isozymes were not changed by IFN-γ (data not shown). The expression levels of ELOVL4 and CerS3 were reduced by IFN-γ in a time- and dose-dependent manner, and maximum suppression was achieved at 14 hours with 50 ng ml^{-1} (Supplementary Figure S3 online). IFN-α, IFN-β, IL-6, IL-22, IL-23, and thymic stromal lymphopoietin had no effect on the mRNA expression of ELOVL4 and CerS3 (data not shown). Cytokines in combination, i.e, IL-4 plus IL-5 and IL-22 plus IL-23, also had no effect on the expression levels of ELOVL4 and CerS3 (data not shown).

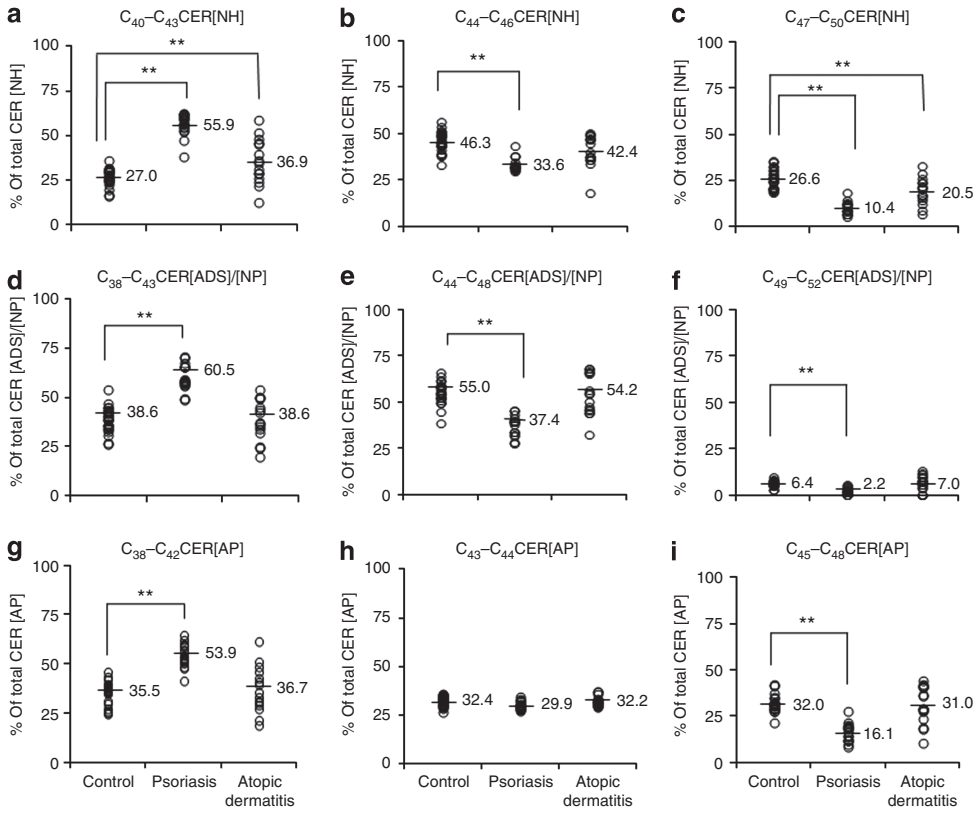

Figure 1. Ceramide (CER) profiles in the stratum corneum (SC) of atopic dermatitis (AD) and psoriasis patients and healthy controls. CER was collected from the SC in the lesional skin of AD patients ($n=15$) and psoriasis patients ($n=15$) and from the normal skin of healthy individuals ($n=22$). CER species of eight classes were identified by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and were sorted into five groups. Each peak was measured, and the relative percentage to total intensity in each group was calculated. Three groups (CER[NH] (**a**–**c**), CER[ADS]/[NP] (**d**–**f**), and CER[AP] (**g**–**i**)) with significant differences are shown. For pair-wise comparisons, Student's *t*-test was used (**$P<0.01$).

IFN-γ reduced mRNA levels of ELOVL and CerS in reconstructed three-dimensional epidermis

In reconstructed three-dimensional epidermis, IFN-γ (100 ng ml^{-1}) reduced the mRNA levels of ELOVL1, ELOVL6, and ELOVL7 by 57.3, 52.0, and 46.0%, respectively (Figure 3a) and those of CerS3, CerS4, and CerS6 by 66.2, 30.9, and 22.8%, respectively (Figure 3b). On the other hand, the mRNA levels of CerS2 and CerS5 were increased by IFN-γ. The mRNA level of ELOVL4 was reduced by IFN-γ at 200 ng ml^{-1} but not at 100 ng ml^{-1} (data not shown).

IFN-γ reduced the amount of CER[NS] with long-chain FA in reconstructed three-dimensional epidermis during SC formation

The amount of CER[NS] was analyzed by ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography (LCMS-IT-TOF). Three-dimensional epidermal sheets on day 7, which possessed minimal SC, were cultured for an additional 7 days in the presence of IFN-γ. By day 14, the three-dimensional epidermal sheets formed multi-layered SC. The amounts of CER[NS] with long-chain FAs, from CER[N(20)S(18)] to CER[N(26)S(18)], were reduced in a dose-dependent manner (Figure 4). Under the same culturing conditions, mRNA levels of ELOVL1 and CerS3 were reduced by IFN-γ in a dose-dependent manner, and this reduction corresponded to the decrease in CER[NS] with long-chain FAs (data not shown).

Signal transducer and activator of transcription (STAT)-independent pathway of IFN-γ mediated downregulation of ELOVL and CerS

To elucidate the IFN-γ signaling pathway involved in CER metabolism, the effect of Janus kinase (JAK) inhibitor Pyridon 6 was examined in NHEKs; Pyridon 6 (1 μM) treatment restored the mRNA levels of ELOVL4 and CerS3, which were suppressed by IFN-γ (Figure 5a and b). Next, to examine the downstream effectors of JAK, STAT1 was knocked down in NHEKs using small-interfering RNA (siRNA). STAT1 mRNA level increased by 3.7-fold in control NHEKs treated with 50 ng ml^{-1} of IFN-γ. In siRNA-treated NHEKs, the basal level of STAT1 mRNA was 24.0% of that in control cells, and IFN-γ-stimulated level was less than the basal level in control cells, confirming that the siRNA was functioning (data

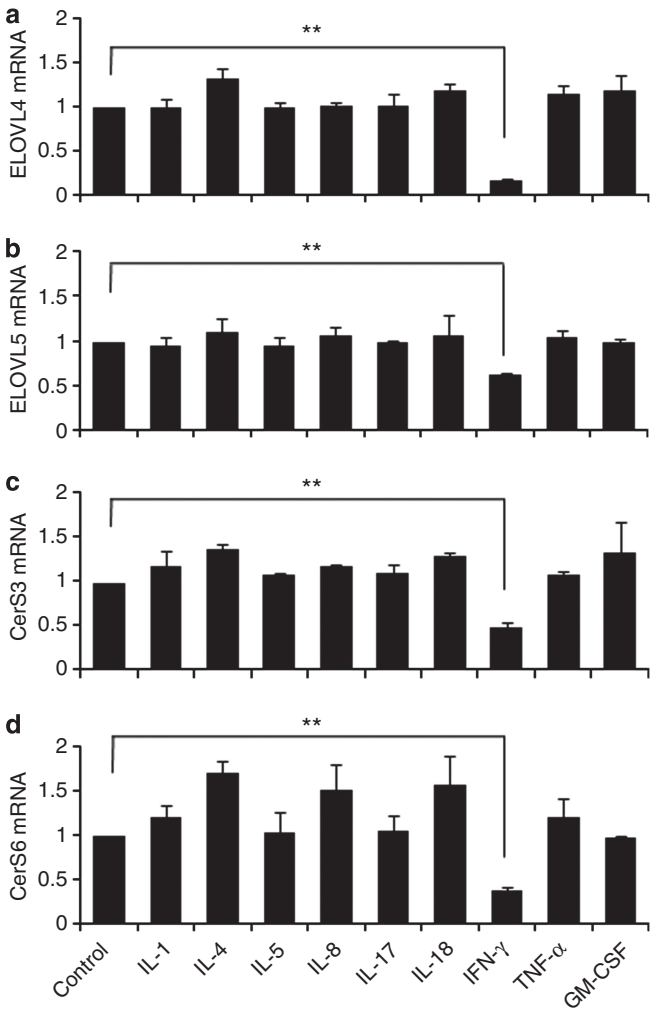

Figure 2. Interferon (IFN)-γ reduced mRNA levels of elongase of long-chain fatty acids (ELOVL) and ceramide synthase (CerS) in normal human epidermal keratinocytes (NHEKs). NHEKs were stimulated with interleukin (IL)-1, IL-4, IL-5, IL-8, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and IFN-γ (50 ng ml^{-1} each), IL-18 (100 ng ml^{-1}), and IL-17 (200 ng ml^{-1}) for 24 hours. Total RNA was extracted and subjected to quantitative reverse-transcriptase–PCR for measurements of (**a**) ELOVL4, (**b**) ELOVL5, (**c**) CerS3, and (**d**) CerS6. Data are presented as mean ± SD (**$P<0.01$).

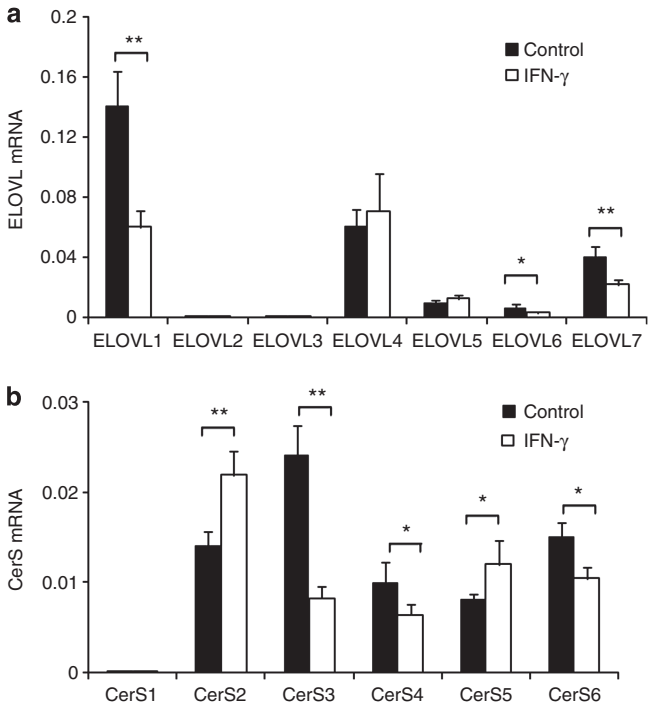

Figure 3. Interferon (IFN)-γ reduced mRNA levels of elongase of long-chain fatty acids (ELOVL) and ceramide synthase (CerS) in reconstructed three-dimensional epidermis. Reconstructed three-dimensional epidermis at day 7 was stimulated with IFN-γ (100 ng ml^{-1}) and incubated for an additional 7 days. Total RNA was extracted and subjected to quantitative reverse-transcriptase–PCR for determining the levels of (**a**) ELOVLs and (**b**) CerSs. Data are relative values to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA and presented as mean ± SD (*$P<0.05$, **$P<0.01$).

not shown). However, suppression of ELOVL4 and CerS3 mRNA expression by IFN-γ was not eliminated by the siRNA treatment (Figure 5c and d). Further, siRNA-mediated knockdown of STAT3 did not affect downregulation of ELOVL4 mRNA expression by IFN-γ (data not shown). Therefore, we examined inhibitors of other factors that could act in cooperation with or independently of STAT1, i.e, MEK (mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) kinase), JNK, p38MAPK, and PI3 kinase (phosphatidylinositol 3′-kinase), but these inhibitors did not affect the IFN-γ-induced downregulation of ELOVL4 mRNA (data not shown).

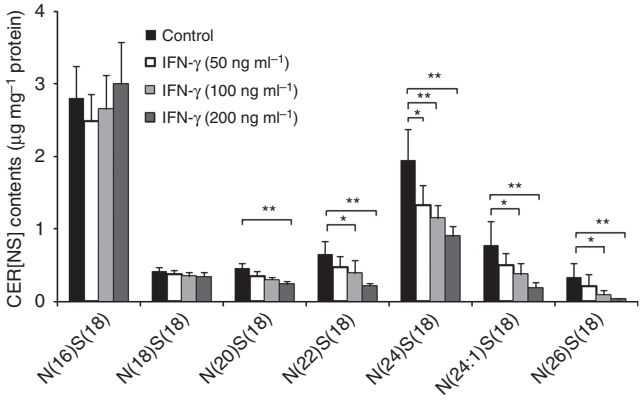

Figure 4. Interferon (IFN)-γ reduced the amount of CER[NS] with long-chain fatty acids in reconstructed three-dimensional epidermis during stratum corneum (SC) formation. Reconstructed three-dimensional epidermis at day 7, which had minimal SC, was cultured in the presence of IFN-γ (50 ng ml^{-1}, 100 ng ml^{-1}, or 200 ng ml^{-1}) for an additional 7 days to form multi-layered SC (day 14). The lipids were extracted from the whole sheets and subjected to LCMS-IT-TOF for determining the levels of each ceramide species. Data are presented as mean ± SD (*P<0.05, **P<0.01).

DISCUSSION

In this study, we first aimed to analyze the lengths of FAs in CERs of AD and psoriasis patients, because CER abnormalities in the SC have been reported for both diseases. Analysis by MALDI-TOF-MS showed significant differences in the FA composition profiles of CERs of the SC between AD and psoriasis patients and normal controls. These differences were detected in specific CER classes: CER[NH], CER[ADS]/[NP], and CER[AP]. However, unexpectedly, significant differences between AD patients and controls were found only in CER[NH], while shortening of FA chain length in other classes of CER has been reported (Ishikawa *et al.*, 2010). The reason for the discrepancy is not well understood. In contrast to AD, significant differences were detected in the FA composition profiles of CERs between psoriasis patients and normal controls in a wide range of CER classes—not only in CER[NH] but also in the CER[ADS]/[NP] and CER[AP] classes. Several studies have investigated the relation between CERs and barrier function in psoriasis. Decreased CER content in

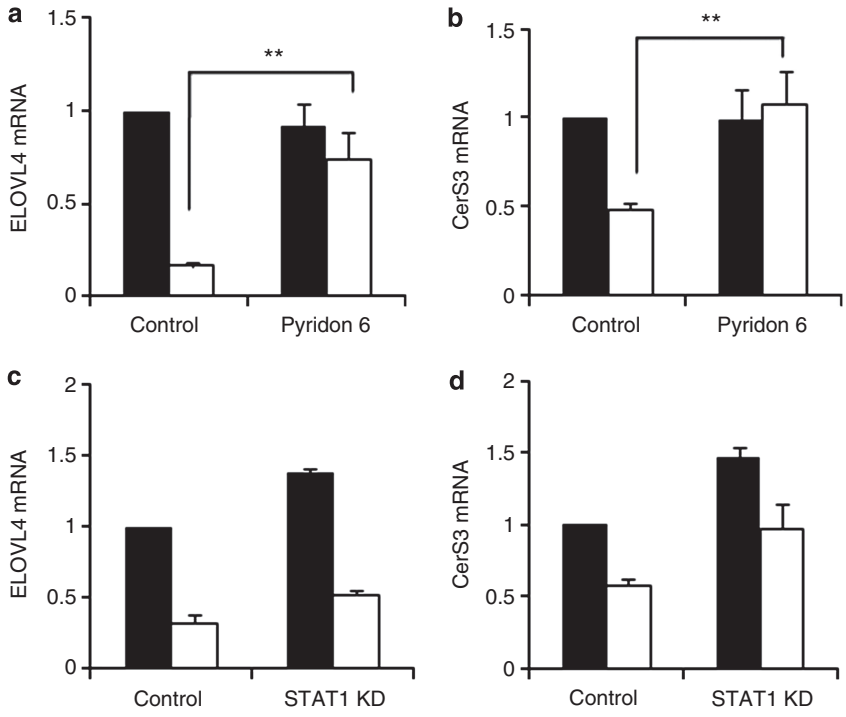

Figure 5. Downregulation of elongase of long-chain fatty acids (ELOVL) and ceramide synthase (CerS) by interferon (IFN)-γ was mediated through a signal transducer and activator of transcription factor 1 (STAT1)-independent pathway. (**a**, **b**) Normal human epidermal keratinocytes (NHEKs) were pretreated with or without 1 μM Janus kinase inhibitor (Pyridon 6) for 1 hour and stimulated with IFN-γ (50 ng ml^{-1}) for 24 hours. (**c**, **d**) NHEKs were treated with or without siRNA against STAT1 for 24 hours and stimulated with IFN-γ (50 ng ml^{-1}) for 24 hours. Total RNA was extracted, and the mRNA levels of ELOVL4 and CerS3 were determined by quantitative reverse-transcriptase–PCR. Data are presented as mean ± SD (**P<0.01). KD, knocked down; closed bar, IFN-γ (−); open bar, IFN-γ (+).

psoriatic skin lesion correlates with increased transepidermal water loss (Motta *et al.*, 1993, 1994). The *de novo* synthesis of CER was significantly lower in lesional epidermis than in non-lesional epidermis and had a negative correlation with the clinical severity of psoriasis (Cho *et al.*, 2004). Although evidence accumulated over the past decade suggests that psoriasis is a T-cell disease, it has been proposed that the epidermal barrier dysfunction caused by disruption of CER synthesis is involved in the pathogenesis of psoriasis (Wolf *et al.*, 2012). To our knowledge, our data provide previously unreported evidence concerning the FA composition of CER in psoriasis, and interestingly, these compositional changes were more pronounced in psoriasis than in AD.

We have found that IFN-γ reduced mRNA expressions of ELOVL and CerS. We have also shown by quantitative analysis of CER[NS] using LCMS-IT-TOF that IFN-γ reduced the amount of CER[NS] with long-chain FAs in reconstructed three-dimensional epidermis. The FA composition differences may be caused by change in the activities of the two enzymes. CERs are synthesized by CerS from a sphingoid base and a fatty acyl-CoA. Although fatty acyl-CoAs with carbon chains up to C16 are synthesized by a FA synthase complex, generation of longer FAs requires ELOVL (Jakobsson *et al.*, 2006). CerS is not involved in FA production *per se*; however, 6 CerS isoforms have been identified, each isoform exhibiting preference for a different fatty acyl-CoA on the basis of FA chain length, suggesting that the enzyme may also affect the FA chain length of CERs (Pewzner-Jung *et al.*, 2006; Mizutani *et al.*, 2009). CerS3 displays a strong affinity for acyl-CoAs with a chain length of C28 or longer, suggesting that CerS3 has an important role in the production of CERs with very long-chain FAs (Rabionet *et al.*, 2008). Of the six CerS isozymes, CerS3 is highly expressed in the skin and is the predominantly expressed isozyme in differentiated human keratinocytes followed by CerS4 (Mizutani *et al.*, 2008). Further, CerS3 deficiency in mice results in complete loss of CER with very long-chain FAs, causing lethal skin barrier impairment (Jennemann *et al.*, 2012). Our results showed that IFN-γ strongly reduced expression of CerS3 mRNA not only in NHEKs but also in reconstructed three-dimensional epidermis. These results suggest that CerS3 may be a key enzyme involved in IFN-γ-induced shortening of the FA chains of CERs in the human skin.

Seven elongases (ELOVL1–7) have been identified in mammals, and each isoform has been shown to exhibit a characteristic substrate specificity. ELOVL1 is important for elongation of saturated long-chain FAs from C20 to C26 (Guillou *et al.*, 2010; Ohno *et al.*, 2010). Knockdown of ELOVL1 in HeLa cells results in reduction of C24 CER and increase in C16 and C18 CERs (Ohno *et al.* 2010). Furthermore, experiments using mice deficient in ELOVL1 have shown that ELOVL1 is a key determinant of FA chain length of CER in epidermis and is essential for permeability barrier formation (Sassa *et al.*, 2013). ELOVL4 is also an essential elongase for the production of very long-chain FAs (Guillou *et al.*, 2010; Uchida, 2011). As in the case of ELOVL1, mice lacking ELOVL4 are unable to produce an adequate amount of long FAs and fail to develop a functionally competent cutaneous permeability barrier (Cameron *et al.*, 2007; Li *et al.*, 2007; Vasireddy *et al.*, 2007). In this study, in reconstructed three-dimensional epidermis, ELOVL1 showed the highest gene expression among the seven isoforms, and IFN-γ significantly reduced ELOVL1 mRNA level. On the other hand, in NHEKs, IFN-γ reduced the expression of ELOVL4 and ELOVL5 but not of ELOVL1. These expression differences may be due to the different cell types; NHEKs mimic basal cells, whereas reconstructed three-dimensional epidermis mimics whole epidermis, which is considered a better physiological model than NHEKs. Therefore, ELOVL1 may be another key enzyme involved in IFN-γ-induced shortening of the FA chains of CERs in the human skin. Taken together, these data strongly suggest that IFN-γ downregulates ELOVL1 and CerS3, which in turn reduces the levels of CERs with long-chain FAs in the human skin.

IFN-γ is one of the most potent proinflammatory cytokines secreted by Th1 lymphocytes. Although recent advances in research have shown that Th17 cells, rather than Th1 cells, are the main modulators in the pathogenesis of psoriasis (Di Cesare *et al.*, 2009; Nestle *et al.*, 2009), increased number of IFN-γ-producing T cells and elevated IFN-γ level have been confirmed in psoriatic lesions, indicating that the predominant presence of IFN-γ is still a characteristic of psoriatic skin lesions (Barker *et al.*, 1991; Szabo *et al.*, 1998; Lowes *et al.*, 2008). In contrast, systemic Th2 imbalance with increased immunoglobulin E levels and eosinophils is widely accepted as the pathogenesis of AD (Del Prete, 1992). However, the inflammation in the skin lesion is biphasic with an initial Th2 phase followed by development of chronic lesions harboring Th1 cells. Furthermore, IFN-γ mRNA and protein are highly expressed in the skin lesions in a vast majority of AD cases (Grewe *et al.*, 1994, 1998; Bieber, 2010). In the present study, we did not provide any direct evidence that IFN-γ downregulates the expression of ELOVL and/or CerS in the skin lesions of psoriasis and AD patients. However, downregulation of ELOVL has been documented in the skin of psoriatic patients by transcriptome analysis (Gudjonsson *et al.*, 2010). In the murine AD model, both downregulation of ELOVL and decreased levels of CER with very long-chain FAs have been reported (Park *et al.*, 2012). Furthermore, we have shown in the present study that change in the FA chain length of CERs was more pronounced in psoriasis than in AD. This may be attributable to the difference in the extent of IFN-γ infiltration in the skin lesions in the two diseases. Thus, it is suggested that IFN-γ may be a key cytokine in the production of CERs with short chain FAs in both AD and psoriasis.

The JAK-STAT cascade is an essential signaling pathway that mediates IFN-γ-induced gene expression. However, recent studies show that IFN-γ initiates signaling cascades, including MEK, JNK, p38MAPK, and PI3 kinase, which may act independently of STAT1 (Ramana *et al.*, 2001; Platanisas, 2005; Gough *et al.*, 2008). Our data show that IFN-γ-induced mRNA suppression of ELOVL4 and CerS3 was abolished by a JAK inhibitor but not by knockdown of STAT1. This indicates that the effects of IFN-γ were mediated through its receptor and not through the STAT1-dependent pathway. As all other signaling inhibitors tested in this study had no effect, we could not decide a specific STAT1-independent pathway in this study.

In conclusion, our results strongly suggest that the IFN-γ-induced suppression of ELOVL1 and CerS3 decreases the levels of CERs with long-chain FAs in the human skin. Thus, the FA profile abnormalities in the CERs of patients with psoriasis and AD may be attributable to the increased IFN-γ levels, which have been reported in the skin lesions of these diseases.

MATERIALS AND METHODS

Nomenclature of CER

CER classes were expressed as the combination of FA residues and sphingoid bases as described previously (Masukawa *et al.*, 2008). Namely, non-hydroxy fatty acid, α-hydroxy fatty acid, ester-linked non-hydroxy fatty acid, ω-hydroxy fatty acid, dihydrosphigosine, sphingosine, phytosphingosine, and 6-hydroxy-sphingosine moieties are designated as N, A, E, O, DS, S, P, and H, respectively, and, for example, non-hydroxy dihydrosphingosine-type CER is expressed as CER[NDS].

Subjects and sample collection from the human skin

This study was approved by the ethical committee of Gifu University Graduate School of Medicine and performed in accordance with the Declaration of Helsinki Principles. Written informed consent was obtained from all volunteers and patients. Samples were collected from 15 subjects with moderate AD (9 men and 6 women; aged 13–72 years; mean age, 32.9 ± 17.3 years), 15 subjects with psoriasis (12 men and 3 women; aged, 42–83 years; mean age, 63.5 ± 14.5 years), and 22 healthy individuals without a history of skin disorders (7 men and 15 women; aged 1–84 years; mean 47.5 ± 23.3 years). To obtain SC specimens, a polyphenylene sulfide film tape (Nichiban, Tokyo, Japan) with an area of 10 mm × 60 mm was pressed on the lesional skin of patients with AD or psoriasis or on the inner forearms of healthy individuals and stripped; this process was repeated 10 consecutive times at the same site. The tapes were stored at −40 °C until lipid extraction.

Lipid extraction from the SC and analysis using MALDI-TOF-MS

Lipid extraction was performed as described previously with a slight modification (Masukawa *et al.*, 2009). The tape was immersed in methanol with sonication for 15 minutes. The lipid extract was dried under a nitrogen stream, dissolved in chloroform, and applied to a Sep-Pak Vac RC silica cartridge (Waters, Milford, MA). The CER-containing fraction was eluted by chloroform/methanol (95:5; v/v), dried under a nitrogen stream, and dissolved in tetrahydrofuran. The solution was mixed with the MALDI matrix solution (1:10; v/v) and subjected to analysis by MALDI-TOF-MS (Bruker Daltonics, Billerica, MA) according to the manufacturer's instruction. Mass spectra obtained were analyzed by peak-picking, and then each peak was identified according to the correspondence table between *m/z* values and all CER species described in the previous report (Masukawa *et al.*, 2009). CER[ADS] and CER[NP], CER[NS] and CER[AH], and CER[NDS] and CER[AS] share most of their *m/z* values with each other, thus they were analyzed as CER[ADS] /[NP], CER[NS]/[AH], and CER[NDS]/[AS], respectively. Intensity of each peak was expressed as the percentage of the total intensity in each CER group. The peak at *m/z* value of 663 was an artifact from the tape used for stripping (Supplementary Figure S1 online). It should be noted that in MALDI analysis quantitative comparisons between samples and between values at different *m/z* points in the same sample are not possible; therefore, only relative compositions of CERs with different *m/z* values are available for comparison between the samples.

Cell culture and reconstructed three-dimensional epidermis

NHEKs isolated from the neonatal skin were purchased from Kurabo (Tokyo, Japan) and cultured in Clonetics KGM-Gold medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract, human epidermal growth factor, recombinant human insulin, hydrocortisone, epinephrine, and transferrin. LabCyte EPI-MODEL (Japan Tissue Engineering, Aichi, Japan) consisting of multilayered foreskin-derived human keratinocytes was used as a reconstructed three-dimensional epidermis, which was cultured in DMEM (Dulbecco's modified Eagle's medium): HamF12 (1:3) supplemented with 5% fetal bovine serum and 1.5 mM $CaCl_2$. After IFN-γ was added, incubation was continued for the indicated period with daily replacement of the culture medium. Whole epidermal sheet was then carefully peeled from the membrane filter of the culture insert and subjected to quantitative reverse-transcriptase–PCR or lipid analysis by LCMS-IT-TOF. All cytokines tested were obtained from Wako (Osaka, Japan). JAK inhibitor Pyridon 6 was purchased from Merck (Billerica, MA).

Lipid extraction from epidermal sheet and analysis by LCMS-IT-TOF

The whole epidermal sheet was lysed by sonication in RIPA (radio-immunoprecipitation assay) buffer supplemented with protease and phosphatase inhibitors, and lipids were extracted from the cell lysates (200 μg proteins) using a modified Bligh and Dyer procedure as described previously (Nemoto *et al.*, 2009). C17-ceramide (Avanti Polar Lipids, Alabaster, AL), which does not exist in the epidermis, was added before lipid extraction as an internal standard for quantification. The lipid extract was then analyzed by LCMS-IT-TOF (Shimadzu, Kyoto, Japan) using the following commercially available standards: CER[N(16)S(18)], CER[N(18)S(18)], CER[N(20)S(18)], CER[N(22)S(18)], CER[N(24)S(18)], and CER[N(26)S(18)] (Avanti Polar Lipids, Alabaster, AL). Chromatographic separations were carried out in a gradient mode using a conventional ODS column (Cadenza CD-C18, 2.0 i.d. × 100 mm; particle size, 3 μm; Imtakt, Kyoto, Japan). MS detection was performed with an electrospray ionization source in positive ion mode.

Quantitative reverse-transcriptase–PCR

Total RNA was isolated from NHEKs or reconstructed three-dimensional epidermis by using an RNA purification kit (NucleoSpin RNA; Macherey-Nagel, Düren, Germany), followed by reverse transcription using the PrimeScript reverse transcriptase, oligo dT, and random primers (all from Takara Bio, Shiga, Japan). Quantitative reverse-transcriptase–PCR was performed in the Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq (Takara Bio). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an endogenous control for data normalization. Sequences of the primers specific for ELOVL and CerS isozymes, STAT1, STAT3, and GAPDH are shown in Supplementary Table S1 online.

siRNA transfection

The siRNAs for STAT1 and STAT3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NHEKs were grown at 40–50% confluence, and siRNA (5 nM) was transfected into the cells by incubating

with the HiPerFect Transfection Reagent (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. After transfection, cells were cultured for further 24 hours until stimulation.

Statistical analysis

All data were expressed as mean ± SD. All experiments were repeated for at least three times. For pair-wise comparisons, Student's *t*-test was used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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