

Decrease of Ceramides with Very Long-Chain Fatty Acids and Downregulation of Elongases in a Murine Atopic Dermatitis Model

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TO THE EDITOR

Ceramide is a family of lipid molecules composed of a sphingoid base and a fatty acid (FA) (Masukawa *et al.*, 2008). Ceramide constitutes a major lipid component of cutaneous permeability barrier, accounting for ~50% of the epidermal membrane structure (Mizutani *et al.*, 2009). Accordingly, the alteration of ceramide in the dermatoses with barrier dysfunction has been a subject of intensive research (Imokawa *et al.*, 1991). Recently, Ishikawa *et al.* (2010) and we (Joo *et al.*, 2010) demonstrated that the profiles of FA lengths of ceramides in the stratum corneum (SC) of atopic dermatitis (AD) patients or people with low cutaneous barrier function (CBF) are different from those with normal CBF. Especially, the ceramides with very long-chain FAs are markedly depleted in SC with impaired CBF and the average chain length of FAs of ceramides are negatively correlated with trans-epidermal water loss (TEWL) in AD patients (coefficient, -0.853 , $P < 0.001$) (Ishikawa *et al.*, 2010), suggesting that synthesis of the FA moiety of ceramides may be dysregulated in AD. Here, we tried to elucidate the mechanism underlying the depletion of ceramides with very long-chain FAs in the SC of AD, by using a hapten-induced AD model in mice.

All animal experiment procedures were approved by the AmorePacific Institutional Animal Care and Use Committee. AD-like skin lesions were induced in female hairless mice with repeated topical applications of 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXZ, Sigma-Aldrich, St Louis,

MO; on day 0 with 50 μ l of 5% to the cervical area and from day 1 to 15 with 100 μ l of 0.1% on bilateral flanks daily). Along with the development of typical AD-like symptoms, including the typical clinical signs of dermatitis (Matsuda *et al.*, 1997), skin thickening, inflammatory cell infiltrations, and epidermal scaling (Figure 1a–c), the impairment of CBF was well simulated as determined by significantly increased TEWL in OXZ-treated mice (Figure 1d). The composition of ceramide[NS] (non-hydroxy acyl sphingosine-type ceramide) (Motta *et al.*, 1993) was quantified as a representative ceramide class in SC samples of control and OXZ-treated mice with a sensitive LC/MS/MS-ESI technique (Joo *et al.*, 2010) to examine the alteration of FA lengths of ceramides. As observed in AD patients (Ishikawa *et al.*, 2010), the ceramide[NS] with very long-chain FAs ($\geq C24$) was markedly reduced in AD-like skin lesions, whereas those with shorter-chain FAs ($\leq C22$) were higher in AD-like skin lesions compared with normal skin (Figure 1e).

To further elucidate a correlation between the respective ceramide[NS] species and their association with AD, principal component analysis, a multivariate analysis that can examine the inter-relationship of multiple factors in an unsupervised manner, was conducted with individual levels of ceramide[NS] species. Remarkably, the principal component 2 (PC2, y-axis) could clearly differentiate the control mice from those treated with OXZ (Figure 2a). Plotting of the loadings of PC2 for the respective ceramide[NS] species showed the apparent separation

of ceramide[NS] species with shorter carbon-chain FAs ($\leq C22$) from those with very long-chain FAs ($\geq C24$) (Figure 2b), reflecting that FA chain lengths of ceramide species may be affected by AD. Ceramides are synthesized by ceramide synthases from a sphingosine base and a fatty acyl-CoA (Mizutani *et al.*, 2009). Fatty acyl-CoAs with carbon chains up to C16 are synthesized by FA synthase complex in mammals, but for the generation of longer FAs ($\geq C18$) membrane-associated FA elongases are required (Jakobsson *et al.*, 2006). At least seven mammalian ELOVL (enzymes for elongation of very long-chain FAs) subtypes (ELOVL1–7) have been identified (Matsuzaka *et al.*, 2002) and skin expresses ELOVL1, ELOVL3, ELOVL4, and ELOVL6, with ELOVL3 limited to the hair follicles and sebaceous glands (Guillou *et al.*, 2010). To investigate the involvement of elongases in the alteration of ceramide[NS] species in AD-like skin lesions, we examined the expression of *Elovl1*, *Elovl4*, and *Elovl6* in skin tissues using RT-PCR. Remarkably, *Elovl1*, *Elovl4*, and *Elovl6* were down-regulated in OXZ-treated mice and *Elovl6* was the least affected (Figure 2c). In line with this, western blot analysis showed that enzyme levels of *Elovl1* and *Elovl4* were lower in OXZ-treated mice (Figure 2d). ELOVL1 and 4 are the essential elongases for the production of very long-chain FAs ($\geq C24$), while ELOVL6 is for medium-chain FAs (C18–C22) from C16 FA (Soupe and Kuypers, 2008). Reduced expression of *Elovl1* and *Elovl4* impairs the elongation of FA beyond C22 and subsequently results in the accumulation of precursor medium-chain FAs ($\leq C22$) (Ofman *et al.*, 2010). Mice lacking a functional copy of *Elovl4* are

Abbreviations: AD, atopic dermatitis; CBF, cutaneous barrier function; FA, fatty acid; OXZ, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; SC, stratum corneum; TEWL, trans-epidermal water loss

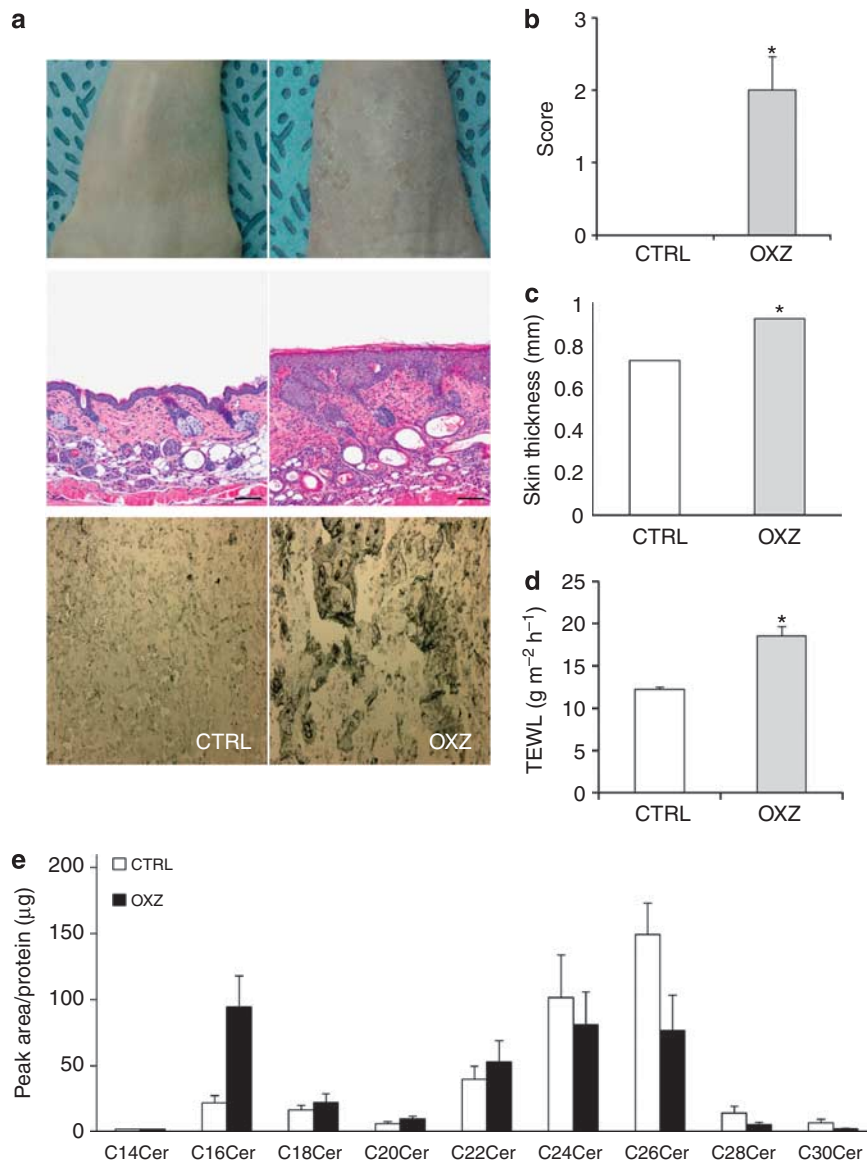


Figure 1. Impaired barrier functions and changes in ceramide species in atopic dermatitis (AD)-like skin lesion. (a) Appearance (upper), histology of skin (middle), and microscopic images of stratum corneum (SC) samples obtained with D-squame tapes (Cuderm, Dallas, TX, diameter, 2.2 cm, lower). Bar = 100 μm. (b) The severity of AD-like symptoms was evaluated by the sum of the individual scores (0, none; 1, mild; 2, moderate; 3, severe) on dryness, excoriation, erythema, and edema. (c) Skin thickness measured as skin-fold thickness with micrometer (Mitutoyo, Kawasaki, Japan). (d) Trans-epidermal water loss (TEWL) measured on the back with Vapometer (Delfin Technology, Kuopio, Finland). (e) Ceramide species were quantified in SC samples collected using five consecutive D-squame tapes from each animal. Tapes were cut into two equal parts and each half was used for lipid extraction and protein assay. Ceramide species were identified by examining fragmentation patterns in liquid chromatography-mass spectrometry (Waters Alliance/Q-Premier XE, Manchester, UK) and comparing with those of commercial standards (C16Cer, C18Cer, C20Cer and C24Cer, Avanti Polar Lipids, Alabaster, AL). Values are expressed as the means ± SE of 5–8 animals per group. *Student's *t*-test between control (CTRL) and 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXZ)-treated mice ($P < 0.05$).

unable to produce an adequate amount of longer FAs ($\geq C26$) and, notably, fail to develop a functionally competent cutaneous permeability barrier (Cameron *et al.*, 2007). In this regard, decreased expression of elongases in AD-like skin lesions and subsequent failure to adequately produce long FAs might have contributed to the depletion

of the ceramide[NS] species with very long-chain FAs that are more essential to CBF than those with shorter FAs (Janusova *et al.*, 2011). Interestingly, peroxisome proliferator activator receptors, liver X receptor, and the sterol regulatory element binding protein signaling pathways, which are important in the transcriptional regulation of

elongases, also have critical roles in epidermal barrier function (Elias, 2005), which may be in line with the alteration of elongase and disrupted CBF in AD-like skin lesions.

In conclusion, we found that the profiles of FA lengths of ceramide species are different in AD-like skin lesions, i.e., the ceramides with very

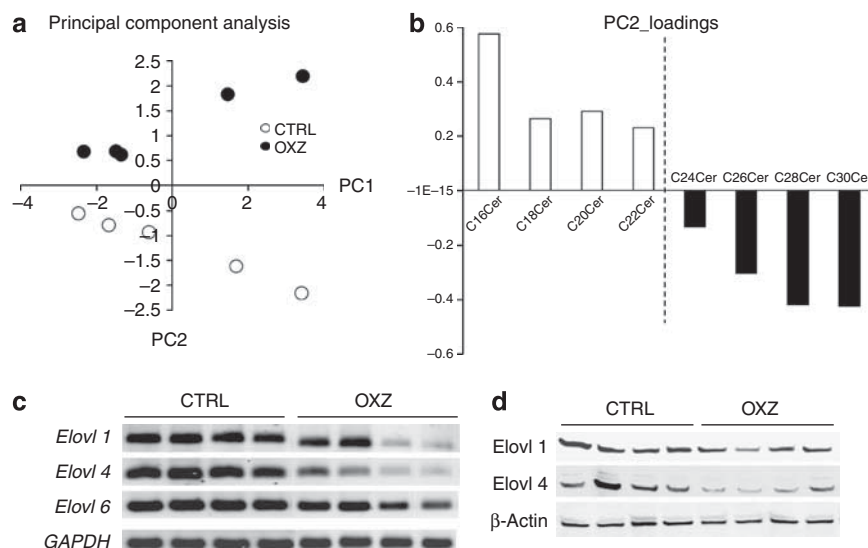


Figure 2. Principal component analysis of ceramide species and the expression of elongases in AD-like skin lesion. (a) Principal component analysis was conducted with the respective contents of ceramide species in individual animals using Minitab statistical software (State College, PA) and a score plot was drawn with PC1 and PC2. The open circles represent five control animals (CTRL) and the filled circles denote five animals treated with 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXZ). (b) The plot of the coefficients of the respective ceramide species for PC2. (c) Dorsal skin biopsy samples were taken and stored in RNAlater RNA stabilization reagent (Qiagen, Germantown, MD) at -80°C . Total RNA was purified from biopsy samples (6 mm ϕ) using RNeasy Plus Mini kit (Qiagen). Complementary DNA of Elov1, 4, 6, and GAPDH was synthesized by RT-PCR and quantitative PCR was performed using SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA). (d) Western blot analysis of Elov1 and 4 in the biopsied skin tissues.

long FAs ($\geq\text{C24}$) decreased whereas those with shorter FAs ($\leq\text{C22}$) increased in AD-like skin lesions. The expression levels of elongases were downregulated in AD-like skin lesions, which might have impaired the synthesis of ceramide species with very long FAs and tilted the balance toward the dominance of ceramide species with shorter FAs over those with longer FAs in the SC of AD. These results suggest that the alteration in elongases as well as in the profile of FA lengths of ceramides could be useful for the diagnosis of AD. More importantly, the modulation of elongases might be effective in repairing damaged CBF in AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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