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Filaggrin knockdown and Toll-like receptor 3 (TLR3) stimulation enhanced the production of thymic stromal lymphopoietin (TSLP) from epidermal layers

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Abstract: Keratinocytes constitute the first-line barrier against exogenous antigens and contain Toll-like receptors (TLRs), which function as pattern-recognition molecules to activate antimicrobial innate immune responses. In an effort to ascertain whether or not filaggrin (*fil*ament-*aggr*egating prote*in*) expression affected the TLR-mediated responses of keratinocytes, we transfected filaggrin siRNA into HaCaT human keratinocyte cells and determined that thymic stromal lymphopoietin (TSLP) and IL-6 secretion were increased by poly(I:C) stimulus. Additionally, TSLP expression is increased in filaggrin knockdown as well as TLR3 stimulation in reconstituted human epidermal layers. Therefore, the findings of this study show that reduced filaggrin levels may influence innate immune responses via TLR stimuli and may contribute to the pathogenesis of inflammatory skin disease via TSLP expression.

Key words: filaggrin – keratinocytes – thymic stromal lymphopoietin – Toll-like receptor

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Background

The skin functions as an indispensible barrier, protecting against a variety of external invaders (1). During infection and injury, pathogenic stimuli are detected by pattern-recognition receptors, specifically via Toll-like receptors (TLRs) within the skin (2). The activation of TLRs on keratinocytes can upregulate proinflammatory cytokine expression via the activation of NF-κB (3).

Filaggrin (*fil*ament-*aggr*egating prote*in*) deficiency or aberrant expression results in phenotypes of Ichthyosis vulgaris and atopic dermatitis, both of which are characterized by disruption of the skin barrier and dry skin (4,5). Such barrier functions are critically important to the immune defense mechanisms of the host (6). Moreover, the pathogenesis of atopic dermatitis may be proposed as a heritable epithelial barrier defect that leads to diminished epidermal defense mechanisms against allergens and microbes, followed by Th2-dominant responses with resultant chronic inflammation. In this study, we attempted to ascertain whether or not the downregulated expression of filaggrin affects TLR-mediated innate immune responses within the epidermal layer.

Questions addressed

We hypothesized that defects in filaggrin potentiate skin irritation caused by infectious agents, such as in cases of atopic dermatitis, via TLR stimulation, and may also influence immune reactions in the direction of Th2-dominant responses. We used RNA interference to achieve filaggrin knockdown in HaCaT cells and in reconstituted the human epidermis and stimulated it with TLR agonist, then subsequently compared the expression of proinflammatory cytokines, IL-1α, IL-1β, IL-6 and thymic stromal lymphopoietin (TSLP).

Experimental design

HaCaT cells (human keratinocyte cell line) were transfected with filaggrin siRNA or control siRNA to reduce endogenous filaggrin expression. Seven hours after transfection, TLR agonists were applied, and the HaCaT cells were maintained for 24 h. The cell lysates and culture supernatants were harvested for immunoblotting and ELISA. We purchased NeoDerm®-E (Tego Science, http://www.tegoscience.com, Korea) grown for 12 days at the air-medium interface and fed the graft with the medium every 4 or 5 days at 37°C, 10% CO_2 and ambient humidity. To induce filaggrin deficiency, the cells were infected with human filaggrin shRNA lentivirus or control with Polybrene. After 24 h of infection, TLR agonists were added and incubated for an additional 2 days for histological and immunological staining. The methods are described in detail in Appendix S1.

Results

Because TLRs play a role in the recognition of microbial components, TLR expression in keratinocytes may affect skin immune responses by enhancing or regulating inflammatory responses via TLR signalling. The results of our RT-PCR analysis showed that HaCaT cells constitutively express mRNA for TLR1 to TLR9, except for TLR7 and 8 (Fig. S1).

Because filaggrin defects might render the skin barrier susceptible to external stimuli, we attempted to determine whether or not filaggrin defects affected the TLR-mediated immune responses of keratinocytes, particularly those occurring as the result of

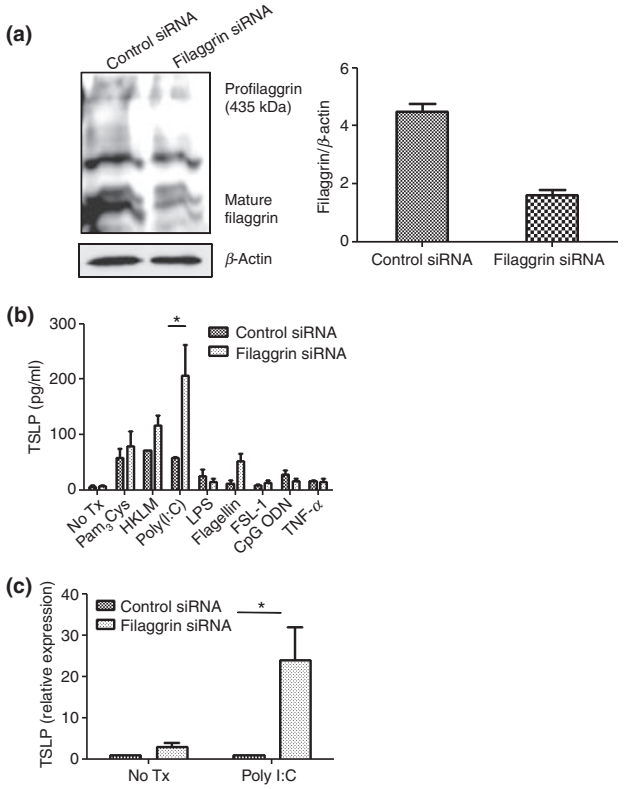

Figure 1. TLR3 stimulation enhances thymic stromal lymphopoietin (TSLP) secretion in filaggrin knockdown HaCaT cells. (a) Filaggrin knockdown in HaCaT cells by siRNA transfection. HaCaT cells at 60–70% confluence were transfected with 80 pmol of human filaggrin or control siRNA using siRNA transfection reagent to reduce endogenous filaggrin expression. Immunoblot of filaggrin in HaCaT cells after 2 days of control or filaggrin siRNA transfection. Beta-actin blotting was used as an endogenous control for proteins in the cells. The data are expressed in ratios of arbitrary pixel density units of filaggrin and β-actin obtained from three independent knockdown experiments (right). (b) Quantification of TSLP from the culture supernatants of TLR agonist-treated HaCaT cells with siRNA transfection. Seven hours after siRNA transfection, TLR agonists were added, and these HaCaT cells were maintained for 24 h. The cell supernatants were harvested for ELISA. TNF-α was employed as a positive control and administered for 24 h at a final concentration of 50 ng/ml. *P* values were analysed via two-way analysis of variance (ANOVA) for significant differences (at $^*P < 0.05$). Data are representative of three independent experiments with $n = 3$ per group and are expressed as the means ± SEM. TLR agonists are Tripamitoyl-S-glyceryl-cysteine (Pam_3Cys, 5 ug/ml) for TLR1, heat-killed *Listeria monocytogenes* (HKLM, 5×10^5/ml) for TLR2, polyriboinosinic polyribocytidylic acid [poly(I:C), 20 ug/ml] for TLR3, lipopolysaccharide (LPS, 10 ug/ml) for TLR4, flagellin (5 ug/ml) for TLR5, Pam2CGDPKHPKSF (FSL-1, 1 ug/ml) for TLR6 and CpG ODN (5 uM) for TLR9. (c) Real-time PCR for TSLP from TLR agonist-treated HaCaT cells with siRNA transfection. *P* values were analysed via two-way ANOVA for significant differences (at $^*P < 0.05$). TLR, Toll-like receptor.

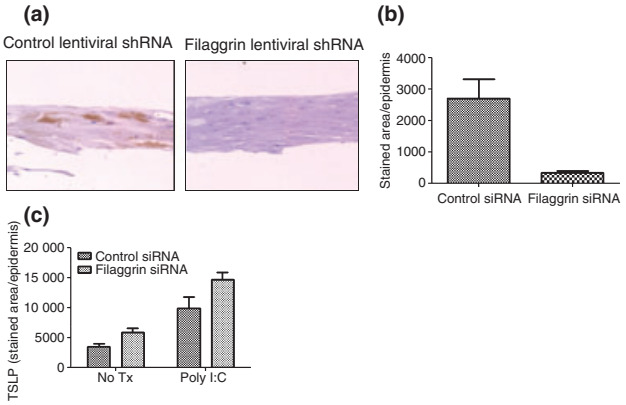

Figure 2. Filaggrin knockdown and poly(I:C) stimulation induce thymic stromal lymphopoietin (TSLP) production in the epidermal layers (NeoDerm®-E). (a) To induce filaggrin deficiency, the cells were infected with human filaggrin shRNA lentiviral particles or control shRNA lentiviral particles with Polybrene in accordance with the manufacturer's instructions. Immunohistochemistry reveals pronounced filaggrin staining in the epidermal layer of the control shRNA-treated group (left), whereas filaggrin was virtually absent in the epidermal layer of filaggrin shRNA-transduced group (right). (b) For the quantification of filaggrin expression, the tissue images were analysed by calculating the filaggrin-stained area (brown area) divided by the area of hematoxylin-stained (pale blue) epidermal layer using AnalySIS software (Soft Imaging System GmbH, Lakewood, CO, USA). Tissues were obtained from three independent batches of shRNA experiments, and three sections per tissue were stained for immunohistochemical analysis. For image analysis, more than 10 image fields were analysed and calculated in each section of the staining. (c) Immunohistochemical analysis of TSLP expression in shRNA-transduced NeoDerm-E® with or without the TLR3 agonist, poly(I:C). To quantify protein expression, the tissue images were analysed with AnalySIS software, as shown in (b). TLR, Toll-like receptor.

proinflammatory cytokine production. To answer this question, we employed the siRNA method to lower the levels of endogenous filaggrin in HaCaT cells and observed a 64% knockdown expression (Fig. 1a). We then attempted to determine whether filaggrin defects enhanced or reduced cytokine production following TLR stimulation. IL-1α is constitutively produced in keratinocytes and released under skin irritation or stimulation conditions and is employed frequently as an early marker for skin irritation (7). Additionally, IL-1α stimulates the further release of inflammatory cytokines, including IL-1α, IL-8, IL-6 and GM-CSF (8). Therefore, we conducted ELISA to determine the expression levels of IL-1α, IL-1β, IL-6 and TSLP. IL-1α secretion was increased by TLR1, TLR2, TLR3, TLR9 and TNF-α stimulation in filaggrin knockdown cells when compared to that observed in the control siRNA group (Fig. S2). Among them, TLR3 stimulation enhanced IL-1β and IL-6 secretion, as well as TSLP secretion in filaggrin knockdown cells (Fig. S2 and Fig. 1b). Additionally, TSLP mRNA was increased only in filaggrin knockdown HaCaT cells as a result of TLR3 stimulation (Fig. 1c).

To analyse the effects of filaggrin knockdown on the epidermal layer, we transduced filaggrin shRNA in NeoDerm®-E layers. Using immunohistochemistry, we confirmed that the expression levels of endogenous filaggrin were lower in the filaggrin shRNA-transduced group than in the control shRNA group (Fig. 2a, b). H&E staining of NeoDerm®-E transduced with either control shRNA or filaggrin shRNA lentivirus revealed no morphological differences (Fig. S3).

Because the mutation of the human filaggrin gene (*FLG*) is associated with atopic dermatitis and TSLP has been shown to be highly expressed in the skin of atopic dermatitis patients, we attempted to determine the relationship between filaggrin knockdown and TSLP production in the epidermal layers. As shown in Fig. 2c, TSLP expression was increased in both the poly(I:C)-treated and filaggrin knockdown groups (Fig. S4).

Conclusions

We determined that reduced filaggrin levels rendered the skin susceptible to inflammatory conditions via the induction of inflammatory cytokines, specifically IL-6 or TSLP, as the result of TLR3-mediated stimulation. TSLP has been previously shown to be abundantly expressed in patients with atopic dermatitis (AD) (9,10). Environmental stimuli trigger keratinocytes for the

production of TSLP, thus activating dendritic cells and mast cells; this results in Th2-type allergic immune responses via the stimulation of DCs and mast cells (11). Besides, filaggrin expression is frequently downregulated in AD patients with no *FLG* mutations owing to the Th2 cytokines, IL-4 and IL-13 (12). Thus, we measured and compared the levels of TSLP expression in filaggrin knockdown epidermal cells. In Fig. 2, TSLP expression in the human epidermal layer was increased under filaggrin knockdown conditions and enhanced in response to poly(I:C) stimuli. This suggests that the filaggrin knockdown conditions induced TSLP secretion and might result in Th2 immune reaction, thus resulting in the propagation of filaggrin reduction in the inflammatory area of the skin of atopic dermatitis patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Detection of TLR mRNA by RT-PCR in cultured HaCaT cells.

Figure S2. Quantification of IL-1α and IL-1β from cellular lysates as well as IL-6 from the culture supernatants of TLR agonist-treated HaCaT cells with siRNA transfection.

Figure S3. H&E staining of NeoDerm-E® transduced with control or filaggrin shRNA with or without TLR3 ligand stimulation.

Figure S4. Immunohistochemical analysis of TSLP expression in shRNA-transduced NeoDerm-E® with or without the TLR3 agonist, poly(I:C).

Appendix S1. Supplementary methods

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Letter to the Editor

Distinct profile of the mitochondrial DNA common deletion in benign skin lesions

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Abstract: Mutations of mitochondrial (mt) DNA, particularly the 4977 bp long common deletion, are increased in aging tissues and preferentially found in chronologically and photoaged skin. Mutations of human mitochondrial DNA (mtDNA) have also been identified in malignant tumors of the skin and of other organs. However, benign skin lesions have not yet been investigated. We analyzed the frequency of the common deletion in 27 benign skin lesions [8 seborrheic keratoses (SK), 5 epidermal nevi (EN), 14 solar lentigos (SL)] by quantitative real-time PCR, because SK and especially SL have been related to (photo)aged skin. All SK and four of five EN displayed reduced common deletion levels compared with adjacent normal skin. In contrast, 50% of SL revealed a higher percentage of the common deletion than the adjacent normal skin, and some SL showed very high absolute common deletion levels up to 14% of total mtDNA. Our results show that the amount of the common deletion is significantly different in benign skin lesions and raise further questions regarding the pathogenesis of SL and its possible role as a precursor lesion of SK.

Key words: common deletion – epidermal nevus – mitochondrial DNA – seborrheic keratosis – solar lentigo

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