

UVB Induces HIF-1 α -Dependent TSLP Expression via the JNK and ERK Pathways

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Thymic stromal lymphopoietin (TSLP) may have a key role in the initiation and maintenance of allergic inflammatory diseases, including atopic dermatitis. The present study revealed that UVB radiation exposure could induce TSLP expression in human keratinocytes and a human skin equivalent model. In addition, we investigated the regulatory mechanism of UVB-induced TSLP expression in keratinocytes. TSLP expression was upregulated by transfection with pcDNA3–hypoxia-inducible factor (HIF)-1 α (P402A and P564A), which stably expresses HIF-1 α protein. UVB-induced TSLP induction in keratinocytes was suppressed in the treatment of mitogen-activated protein kinase inhibitors or small interfering RNAs against HIF-1 α . The results of chromatin immunoprecipitation assays indicate the direct involvement of HIF-1 α in UVB-mediated TSLP induction. Taken together, these findings indicate that UVB exposure may increase TSLP expression through a HIF-1 α -dependent mechanism via the c-JUN N-terminal kinase and extracellular signal-regulated kinase pathways in human keratinocytes. Our data showed that UVB-induced TSLP might increase secretion of the T-helper type 2–attracting chemokine (c–c motif) ligand 17 by human dendritic cells. The present study suggests an important role of HIF-1 α in UVB-mediated immune response in keratinocytes.

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INTRODUCTION

As an environmental factor, UVB radiation has multiple effects on human health. UVB light is an electromagnetic radiation with a wavelength from 280 to 320 nm. It is well known that UVB exposure is required to produce vitamin D in the skin (Holick, 2003). Meanwhile, UVB radiation can cause sunburn, some forms of skin cancer (Melnikova and Ananthaswamy, 2005), and immune responses (Paz *et al.*, 2008; Gonzalez Maglio *et al.*, 2010) by inducing abnormal cytokine release in keratinocytes. However, the molecular mechanism involved remains unknown.

Elevated expression of thymic stromal lymphopoietin (TSLP) was recently reported in skin diseases, such as atopic dermatitis (Soumelis *et al.*, 2002). TSLP is an IL-7-like

T-helper type 2 (Th2)–promoting cytokine and represents a critical factor linking responses at interfaces between the body and environment to allergic type 2 immune responses (Vu *et al.*, 2011). The main sites of TSLP production are epithelial cells and keratinocytes; however, other cells such as smooth muscle cells, allergen-activated basophils, and IgE-primed mast cells can also express TSLP (Soumelis *et al.*, 2002). A variety of environmental factors (e.g., viruses, microbes, allergen sources, and chemicals), proinflammatory cytokines (e.g., IL-1 β and tumor necrosis factor- α), and selected Toll-like receptor agonists induce TSLP production (Kouzaki *et al.*, 2009; Hong *et al.*, 2011; Kawasaki *et al.*, 2011; Redhu *et al.*, 2011; Vu *et al.*, 2011; Satou *et al.*, 2012). However, it remains unknown whether UVB induces TSLP expression in the skin. To determine the effect of UVB exposure on TSLP expression in the skin, we examined TSLP expression in human keratinocytes and a human skin equivalent model (HSEM) after exposure to UVB.

Hypoxia-inducible factor-1 α (HIF-1 α), a transcriptional factor that regulates the expressions of various proteins, especially under hypoxia, is known to induce cytokine expression under pathological conditions (Egger *et al.*, 2007). Recent reports suggest that HIF-1 α may be an important regulator in the keratinocytes response to UVB exposure (Rezvani *et al.*, 2007; Wunderlich *et al.*, 2008). Previous studies showed that UV (290–400 nm)-induced HIF-1 α might be a factor to suppress T cell-mediated immunity (Cho *et al.*, 2012). In addition, there is increasing evidence about the role of HIF-1 α in allergic inflammation. HIF-1 α regulates vascular permeability via vascular endothelial growth factor (VEGF)

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Abbreviations: CCL17, chemokine (c–c motif) ligand 17; ChIP, chromatin immunoprecipitation; DC, dendritic cell; ERK, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; HSEM, human skin equivalent model; JNK, c-JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; Th, T helper; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor

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in an allergic airway disease (Lee *et al.*, 2008) and also has a crucial role in adaptive Th2-mediated inflammation (Sumbayev *et al.*, 2009). However, it remains unclear whether HIF-1 α activation contributes to the induction of UVB-induced TSLP. The present study suggests that HIF-1 α is an important regulator of TSLP expression in keratinocytes after UVB exposure.

At the molecular level, UVB radiation modulates intracellular signaling pathways that affect gene expression. The mitogen-activated protein kinase (MAPK) family is fundamental in mediating numerous changes in cell functions, such as cytokine expression, proliferation, and apoptosis (Peus *et al.*, 1999; Puddicombe and Davies, 2000). To elucidate the intracellular mechanism that regulates TSLP expression upon UVB irradiation, we examined the activation of MAPKs on HIF-1 α and TSLP expression using inhibitors.

The present study shows that UVB irradiation can induce TSLP expression in human keratinocytes via HIF-1 α stabilization, which is dominantly mediated via the c-JUN N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways.

RESULTS

Upregulation of TSLP expression by UVB irradiation in human keratinocytes

To determine whether UVB induces TSLP expression, HaCaT keratinocytes were irradiated with different UVB doses (0, 10, 50, 100, and 200 mJ/cm²) and incubated for 24 hours. In UVB-irradiated HaCaT cells, TSLP mRNA was upregulated in a dose-dependent manner (Figure 1a). As 100 mJ/cm² UVB irradiation did not cause cell death, this amount was selected for subsequent experiments (data not shown). TSLP mRNA expression increased up to 1.5-fold in cells irradiated with 100 mJ/cm² UVB compared with the control. Time-dependent increases in corresponding protein expression were also detected on cell extracts (Figure 1b). An ELISA was performed to evaluate the secretion level of TSLP in the culture supernatant of HaCaT cells. Consistent with elevated TSLP protein expression, TSLP secretion was also elevated after UVB exposure in a dose-dependent manner (Figure 1c). In addition, we investigated the production of UVB-induced TSLP in normal human epithelial keratinocytes. These results were consistent with the results in HaCaT cells (Supplementary Figure S1 online). To confirm the levels of TSLP expression induced by UVB in human skin, we used a HSEM. HSEMs are similar to normal human epidermis and consist of multi-layered, differentiated tissue, including basal, spinous, granular, and cornified layers (Curren *et al.*, 2006). TSLP expression was higher in the UVB-irradiated HSEM than in the nontreated HSEM (Figure 1d). To investigate the biological significance of UVB-induced TSLP, we investigated chemokine (c-c motif) ligand 17 (CCL17) secretion by human dendritic cells (DCs). DCs were treated for 48 hours in the absence or presence of conditioned media of UVB-irradiated keratinocytes with or without neutralizing polyclonal antibody against TSLP. Our results showed that CCL17 secretion by DCs was significantly increased in conditioned media-treated cells compared with the control (Figure 1e). In contrast, CCL17

expression in conditioned media-treated cells was neutralized by polyclonal antibody against TSLP (Figure 1e). These findings suggest that UVB-induced TSLP may increase the production of the Th2-attracting chemokine CCL17 by human DCs.

UVB-induced HIF-1 α stabilization in human keratinocytes

To confirm that HIF-1 α is involved in the UVB-induced response in human keratinocytes, we first examined HIF-1 α expression caused by UVB irradiation in HaCaT keratinocytes. The mRNA expression level of HIF-1 α was not upregulated by UVB (Figure 2a), but its protein expression level increased from 16 hours after UVB radiation (Figure 2b). As HIF-1 α functions as a transcriptional factor after translocation into the nucleus, UVB-irradiated HaCaT keratinocytes were fractionated into the cytoplasm and nuclei, and the HIF-1 α protein level in each fraction was compared. HIF-1 α protein was hardly detected in the cytoplasmic fraction of UVB-irradiated human keratinocytes and the control. However, nuclear-translocated HIF-1 α protein levels were higher in UVB-irradiated human keratinocytes than in the controls (Figure 2c). These results indicate that UVB induces the stabilization of HIF-1 α , leading to its nuclear translocation.

Mediation of UVB-induced TSLP upregulation by HIF-1 α in human keratinocytes

To definitively demonstrate the role of HIF-1 α in TSLP induction, we analyzed the effect of HIF-1 α on TSLP expression by HIF-1 α gene manipulation. To constitutively express HIF-1 α in HaCaT keratinocytes, we utilized pcDNA3-HIF-1 α , which stably expresses HIF-1 α protein by amino acid replacement (P402A and P564A) on the hydroxylation site (Tal *et al.*, 2008). Compared with that in the control, in the transfected keratinocytes with pcDNA3-HIF-1 α , TSLP expression increased as a result of exogenous HIF-1 α upregulation at the mRNA (Figure 3a) and protein levels in (Figure 3b). To confirm the HIF-1 α dependency of TSLP induction, we analyzed the effect of HIF-1 α silencing on the expression of TSLP mRNA due to UVB. UVB-induced TSLP levels were downregulated by HIF-1 α small interfering RNA (siRNA) as opposed to that in the control (Figure 3c). Taken together, these results suggest that HIF-1 α contributes to the regulation of TSLP expression in human keratinocytes.

Mediation of UVB-induced HIF-1 α stabilization by JNK and ERK phosphorylation in human keratinocytes

UVB is reported to activate the MAPK signaling cascade, which includes the JNK, ERK, and p38 signaling pathways, which are known to regulate HIF-1 α expression (Rezvani *et al.*, 2007). On the basis of previous reports, the expression and phosphorylation of corresponding proteins were examined in UVB-irradiated HaCaT keratinocytes. JNK phosphorylation mainly increased 2 hours after UVB irradiation. ERK phosphorylation began 2 hours after UVB irradiation and was maintained at a similar level up to 12 hours. Similar to JNK, p38 phosphorylation started to increase at 2 hours and peaked 4 hours after irradiation

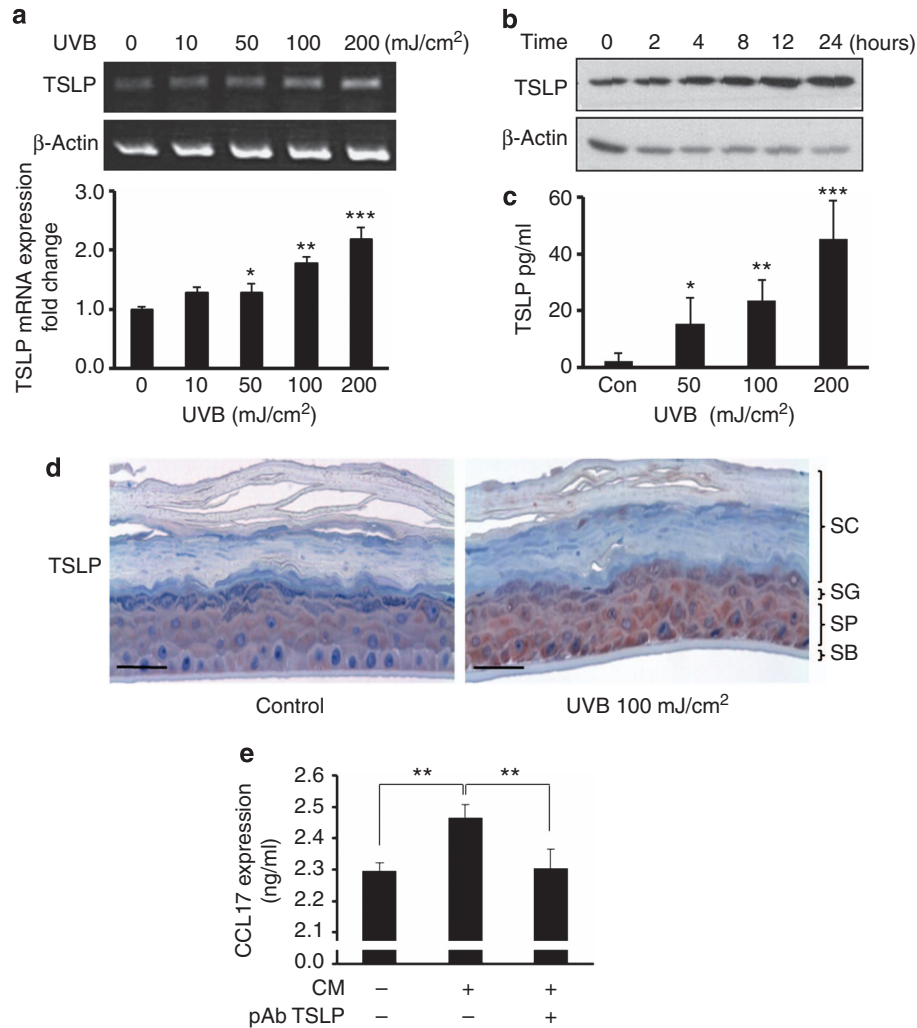


Figure 1. Upregulation of thymic stromal lymphopoietin (TSLP) expression by UVB irradiation in human keratinocytes. (a) TSLP mRNA levels were upregulated by UVB irradiation in a dose-dependent manner. (b) Elevated TSLP protein expression was detected in a time-dependent manner. (c) UVB irradiation increased TSLP protein secretion in a dose-dependent manner. Secreted TSLP levels in culture supernatant were measured by ELISA. (d) UVB irradiation promoted TSLP expression in the human skin equivalent models (HSEMs). (e) UVB-induced TSLP increased chemokine (c-c motif) ligand 17 (CCL17) secretion by dendritic cells (DCs). CCL17 production in culture supernatant of DCs was determined by ELISA. Bar = 50 μ m. SB, stratum basale; SC, stratum corneum; SG, stratum granulosum; SP, stratum spinosum. Asterisks indicate statistical significance at * P <0.05, ** P <0.01, and *** P <0.001.

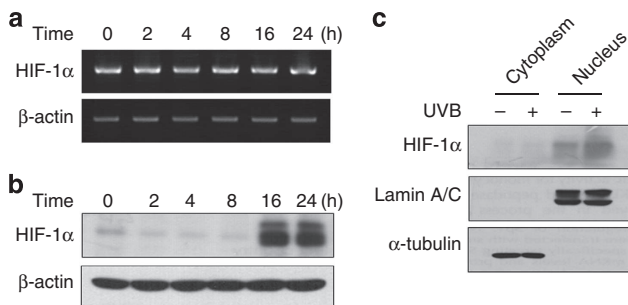


Figure 2. UVB-induced hypoxia-inducible factor (HIF)-1 α stabilization in human keratinocytes. (a) HIF-1 α mRNA was not upregulated by UVB irradiation. (b) HIF-1 α protein expression increased 16 hours after UVB irradiation. (c) UVB irradiation promoted the nuclear translocation of HIF-1 α protein. Nuclear and cytoplasmic fractions were prepared 24 hours after UVB irradiation. HaCaT keratinocytes were irradiated with 100 mJ/cm² UVB.

(Figure 4a). To further investigate the effect of MAPKs on UVB-induced HIF-1 α expression, we treated cells with JNK, ERK, or p38 inhibitors before UVB irradiation. In particular, the inhibition of phosphorylated JNK and ERK significantly decreased HIF-1 α expression, which was subsequently associated with reduced TSLP expression in UVB-irradiated keratinocytes (Figure 4b). These results suggest that UVB-induced HIF-1 α and TSLP expression can be mediated via the JNK and ERK pathways in human keratinocytes.

Regulation of TSLP expression through the binding of HIF-1 α to the promoter of TSLP in human keratinocytes

We performed a luciferase assay to confirm whether UVB affects TSLP promoter activity. UVB irradiation increased the luciferase activity of TSLP promoter-pGL3 by approximately threefold compared with that in the control (Figure 5a).

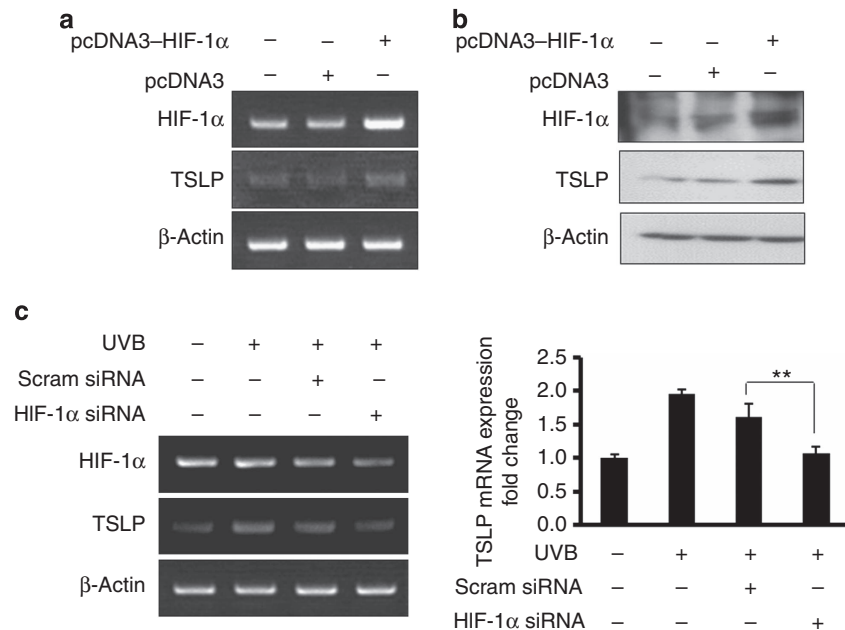


Figure 3. Mediation of UVB-induced thymic stromal lymphopoietin (TSLP) upregulation by hypoxia-inducible factor (HIF)-1α in human keratinocytes. (a) TSLP expression was increased by exogenous HIF-1α upregulation at the mRNA and (b) protein levels. To induce the constitutive expression of HIF-1α, pcDNA3-HIF-1α (P402A and P564A) expression vector was transfected into HaCaT keratinocytes, which were then incubated for 24 hours. (c) UVB-induced TSLP expression was downregulated by HIF-1α small interfering RNA (siRNA). At about 40% confluence, cells in 60-mm cell culture dishes were transfected with 20 nM siRNA and then allowed to stabilize for 24 hours before being used in experiments. Scram, scrambled. Asterisks indicate statistical significance at $**P < 0.01$.

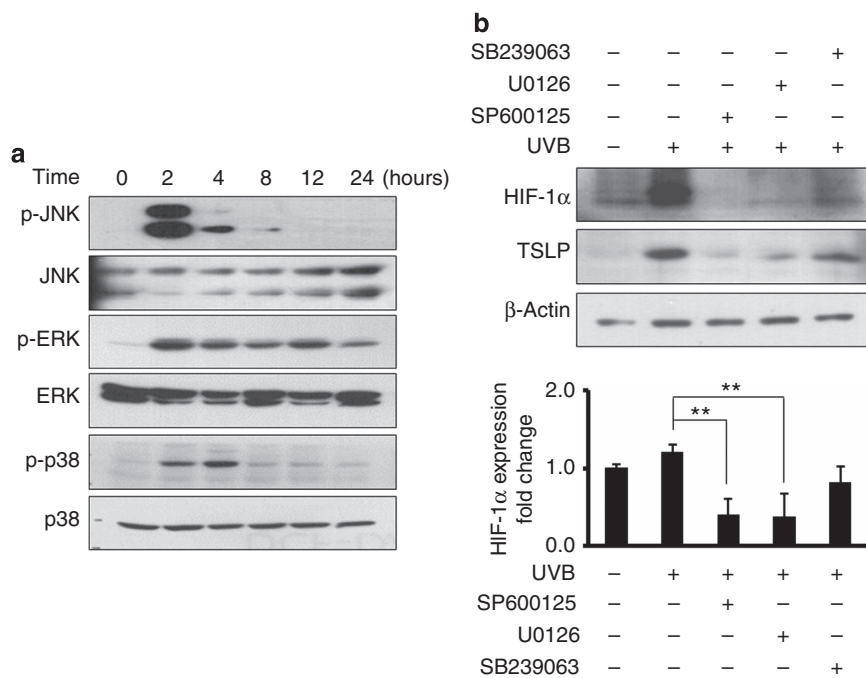


Figure 4. Mediation of UVB-induced thymic stromal lymphopoietin (TSLP) upregulation by c-JUN N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) phosphorylation in human keratinocytes. (a) UVB increased mitogen-activated protein kinase (MAPK) phosphorylation in HaCaT keratinocytes. Cells were collected at the different time points after UVB irradiation. JNK, ERK, and p38 were phosphorylated 2 hours after UVB irradiation. (b) Inhibition of MAPKs diminished UVB-induced HIF-1α and TSLP protein expressions. Before UVB irradiation, the cells were pretreated with SP600125, U0126, and SB239063 JNK, ERK, and p38 inhibitors, respectively. Asterisks indicate statistical significance at $**P < 0.01$.

In addition, we performed a luciferase assay using HIF-1α overexpression to determine whether HIF-1α activates the TSLP promoter. In the absence of UVB irradiation, HIF-1α

overexpression increased the luciferase activity of TSLP promoter-pGL3 by approximately twofold compared with that in the control (Figure 5b).

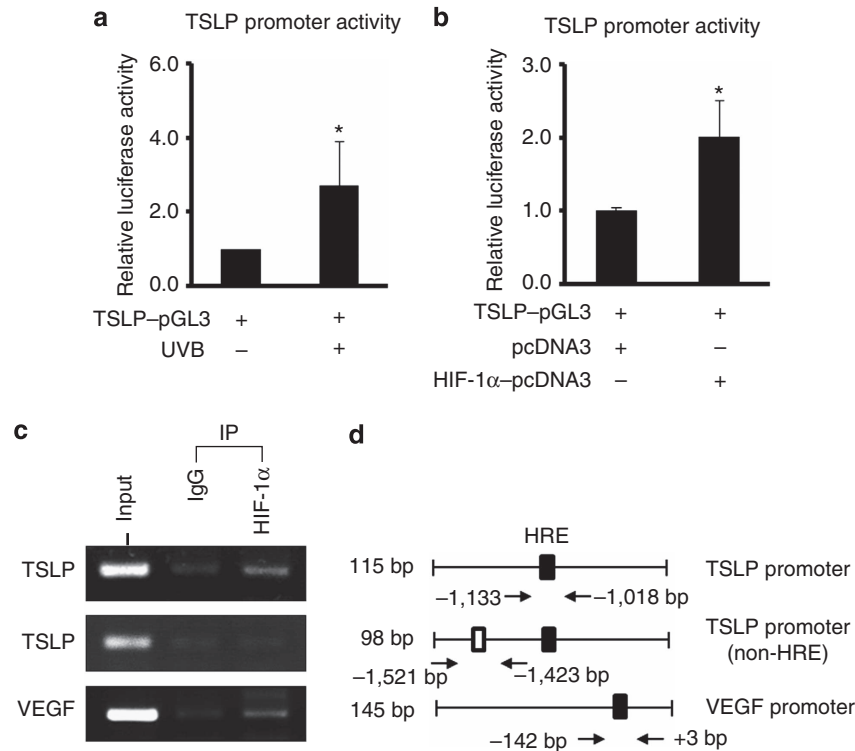


Figure 5. Regulation of thymic stromal lymphopoietin (TSLP) expression by hypoxia-inducible factor (HIF)-1 α through its binding to the TSLP promoter in human keratinocytes. (a) UVB activated the TSLP promoter in HaCaT keratinocytes. (b) HIF-1 α activated the TSLP promoter. (c) HIF-1 α bound the hypoxia response element (HRE) site of the TSLP promoter. HaCaT keratinocytes were irradiated with UVB and then incubated for 24 hours. Chromatin complexes were immunoprecipitated with anti-HIF-1 α antibody, and eluted DNA was then amplified by PCR using specific primers. Primers for vascular endothelial growth factor (VEGF) promoter were used as positive control. (d) Arrows show the positions and directions of PCR primers used for detecting the products of the chromatin immunoprecipitation (ChIP) assay. Asterisks indicate statistical significance at * $P < 0.05$.

We performed chromatin immunoprecipitation (ChIP) assays to verify our assumption that HIF-1 α directly binds and regulates the TSLP promoter in human keratinocytes. HaCaT keratinocytes were irradiated with UVB and incubated for 24 hours. We detected specific amplification of the TSLP promoter region after a ChIP assay using anti-HIF-1 α antibody. As a positive control, we used specific primers spanning the VEGF promoter region, which are well-known targets of HIF-1 α . Specific bands corresponding to TSLP and VEGF were detected when using human genomic DNA. To exclude the nonspecific binding of antibodies, chromatin complexes were also immunoprecipitated with anti-IgG antibody as a negative control. No specific amplification was observed in the presence of a nonrelevant mouse antiserum (Figure 5c). The arrows in Figure 5d show the positions and directions of the PCR primers used for detecting the products of the ChIP assay. Taken together, these results suggest that the role of HIF-1 α in TSLP expression is mediated by the direct control of transcriptional activity of the TSLP promoter in human keratinocytes.

DISCUSSION

The UVB-induced immune response usually begins with an abnormal release of inflammatory cytokines, such as IL-1, IL-6, IL-8, and tumor necrosis factor- α , in human keratinocytes (Kondo *et al.*, 1993; Kulms *et al.*, 2000). UV radiation is also

immunosuppressive (Ullrich, 2007). UV-induced prostaglandin E2 induces the production of IL-4 and IL-10, which then cause DCs to secrete immune suppressive IL-12p40 homodimers (Schmitt and Ullrich, 2000). It has been reported that platelet-activating factor can upregulate the production of prostaglandin E2 and be a crucial factor in UV-induced immune suppression (Wolf *et al.*, 2006).

Recent papers showed that UVB irradiation might upregulate Th2-type cytokines, such as IL-31 and IL-33 (Cornelissen *et al.*, 2011; Meehansan *et al.*, 2012). Through skin biopsies from healthy human volunteers who were exposed to UVB, Di Nuzzo *et al.* (2002) showed that UV-irradiated skin could provide a microenvironment to favor the development of Th2-type T cells. In addition, Walters *et al.* (2003) also demonstrated that therapeutic UVB suppressed the production of INF- γ , IL-2, and IL-12, and increased IL-4, which together could account for a shift of Th2 immune response. The shift toward a Th2 immune response may be one of the major factors determining the therapeutic efficacy of UVB phototherapy (Berneburg *et al.*, 2005). However, the actual pathways underlying photobiology are not known in details.

TSLP is an IL-7-like cytokine belonging to the Th2 cytokine family and has a key role in allergic inflammatory diseases. TSLP acts as a master switch for Th2 immune responses via the activation of DCs and mast cells. According to previous studies, TSLP is mainly observed in the lesions of atopic

dermatitis patients and bronchial epithelial cells in asthma patients (Soumelis *et al.*, 2002; Ying *et al.*, 2008). TSLP-activated DCs secrete Th2-recruiting chemokines, which cause naive T cells to differentiate into inflammatory Th2 cells producing IL-4, IL-5, IL-13, and tumor necrosis factor- α via the OX40 ligand (Ito *et al.*, 2005; Allakhverdi *et al.*, 2007). Therefore, understanding the environmental factors and regulatory mechanism of TSLP induction may be essential for clarifying the pathogenesis of allergic diseases. In this study, we showed that UVB exposure can induce TSLP expression in keratinocytes. Interestingly, in addition to allergic inflammatory diseases, TSLP exerts a cancer-promoting effect via the TSLP receptor on CD4+ T cells, which causes the promotion of a Th2-skewed immune response and production of immunosuppressive factors, such as IL-10 and IL-13 (Olkhanud *et al.*, 2011). Thus, the present findings would be helpful for better understanding the pathogenesis of UVB-mediated skin cancer.

TSLP-activated DCs can induce the production of the Th2-attracting chemokine CCL17, also known as TARC (thymus and activation-regulated chemokine) (Soumelis *et al.*, 2002). The present study showed that UVB-induced TSLP might increase CCL17 secretion by human DCs. These findings demonstrate that UVB irradiation may induce Th2-type immune response by producing TSLP in human keratinocytes. However, the interplay of the various photobiological responses is still complex and not completely understood. It will be of interest to further explore the biological effects of UVB-induced TSLP in cutaneous dermatoses.

As HIF-1 α is upregulated by UVB and associated with cytokine production in pathological states, its transcriptional regulation of UVB-induced TSLP production was also examined. In the present study, UVB exposure promoted TSLP expression in a dose-dependent manner, which was mediated by HIF-1 α accumulation. At the transcription level, NF- κ B and AP-1 are known to be regulators of inflammation-induced TSLP expression (Vu *et al.*, 2011). However, the present study examined HIF-1 α , another transcriptional factor activated by UVB, and we hypothesized that HIF-1 α regulates UVB-induced TSLP expression in human keratinocytes. Sequence-based analysis using TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) revealed that the TSLP promoter contains a hypoxia response element that allows HIF-1 α to bind. Besides, it is reported that HIF-1 α has an important role in inflammatory responses (Lee *et al.*, 2006). As expected, increased HIF-1 α protein accumulation, induced by HIF-1 α protein stabilization, was observed in human keratinocytes after UVB irradiation (Figure 2b). ERK, JNK, and p38 MAPK are reported to be involved in the regulation of HIF-1 α expression in cells under stress, such as hypoxia and UVB radiation (Rezvani *et al.*, 2007). In particular, consistent with previous reports, UVB-induced ERK and JNK phosphorylation in human keratinocytes that mediates HIF-1 α expression in human keratinocytes. Using MAPK inhibitors, we showed that ERK and JNK phosphorylation is required for HIF-1 α accumulation in keratinocytes after UVB exposure.

We confirmed the association between HIF-1 α and TSLP induction by analyzing the effect of HIF-1 α on TSLP

expression using *HIF-1 α* gene manipulation. HIF-1 α overexpression and ChIP assays further demonstrated that HIF-1 α directly binds the hypoxia response element consensus sequence located at position -1,060 of the TSLP promoter and regulates the transcriptional activity of TSLP. The present results suggest that HIF-1 α is a transcriptional regulator of TSLP in human keratinocytes after UVB irradiation.

Taken together, the present study indicates that UVB exposure increases TSLP expression by HIF-1 α activation via the JNK and ERK pathways in human keratinocytes. These findings suggest an important role of HIF-1 α in UVB-irradiated skin.

MATERIALS AND METHODS

Reagents

The antihuman TSLP polyclonal antibody was purchased from Abcam (Cambridge, MA). The antihuman HIF-1 α mAb was purchased from BD Biosciences (San Diego, CA). The anti-lamin A/C, anti-phospho-ERK/ERK, anti-phospho-JNK/JNK, and anti-phospho-p38/p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the anti- β -actin and anti- α -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SP600125 and U0126 were purchased from Sigma-Aldrich (St Louis, MO) and SB239063 was purchased from Cell Signaling Technology. Neutralizing polyclonal antibody against human TSLP was purchased from R&D Systems (Minneapolis, MN).

Cell cultures

The human immortalized HaCaT keratinocyte cell line was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and maintained in DMEM (HyClone, Logan, UT), containing 10% fetal bovine serum (HyClone), penicillin 100 IU/ml, and streptomycin 100 μ g/ml (GIBCO, Carlsbad, CA). Normal human epithelial keratinocytes were purchased from Millipore (Temecula, CA) and cultured in human epidermal keratinocyte complete media kit (EpiGRO; Millipore). DCs were purchased from MatTek Corporation (DC-100; Ashland, MA) and cultured in complete media (MatTek Corporation). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂. The human reconstituted epidermis 3D EpiDerm models (EPI-200), which were used as HSEMs, were purchased from MatTek Corporation. HSEMs were incubated in assay medium at 37 °C and 5% CO₂ and used for immunohistochemistry analysis.

UVB irradiation

A UVB lamp (PL-S 9W/01/2P; Philips, Warszawa, Poland) with a 280- to 320-nm emission was used. The UVB fluence rate was simultaneously measured and integrated using a radiometer (UVB-500C; National Biological, Twinsburg, OH) placed at the same distance from the UVB source. A dose of 100 mJ/cm² was used throughout the study, because this dose yielded the optimal effect without significantly affecting cell viability. Control cells were subjected to the identical procedure without being exposed to UVB.

Reverse transcription-PCR

Total RNA was extracted from UVB-irradiated human keratinocytes using the TRIzol Reagent (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Reverse-transcription reactions were performed using 1 μ g total RNA. The following primers were used: TSLP:

sense 5'-TAGCAATCGGCCACATTGCCT-3', antisense: 5'-GAAGC GACGCCACAATCCTTG-3'; HIF-1 α : sense 5'-GATTTTGGCAG CAACGACAC-3', antisense 5'-TGAATCTGGGGCATGGTAAA-3'; β -actin: sense 5'-ACAGGAAGTCCCTTGCCATC-3', antisense 5'-AGG GAGACCAAAAGCCTTCA-3'. Amplification was performed on a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA).

Nuclear and cytoplasmic extract isolation and western blotting

Nuclear and cytoplasmic extracts were prepared with the Nuclear Extract Kit (Active Motif, Tokyo, Japan), and total protein was extracted from cells with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 μ g/ml phenylmethanesulfonylfluoride, and protease inhibitors). Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) using BSA as the standard. Protein lysates (30 μ g) were resolved by 10–12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). After blocking, the polyvinylidene difluoride membranes were incubated overnight with indicated primary antibodies on a shaker at 4°C. After extensive washing, the membranes were incubated with secondary peroxidase-linked IgG (1:5,000, Abcam) for 1 hour. After washing three times for 10 minutes with Tris-buffered saline with Tween 20 at room temperature, the immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Piscataway, NJ).

ELISA

Immunoreactive TSLP and CCL17 within the supernatants were quantified using DuoSet TSLP ELISA assays (R&D Systems) and TARC/CCL17 ELISA assay kit (Abcam), according to the manufacturer's instructions.

DNA construction

To induce constitutive HIF-1 α expression regardless of oxygen tension, the oxygen-dependent degradation domain was mutated in complementary DNA encoding HIF-1 α , as previously described (Tal *et al.*, 2008). To generate the pcDNA3-HIF-1 α (P402A and P564A) expression vector, the DNA fragments encoding the human HIF-1 α (P402A and P564A) mutant were amplified using the following set of primers: 5'-GCAGATCTATGCCGACGGTGGAGGAGC-3' and 5'-GCCGAATTCGATTTCCTGAAGGCTC-3'. The amplified DNAs were subsequently cloned into the *Bgl*II/*Eco*RI sites of the pcDNA3 vector (Invitrogen). For the TSLP promoter assay, the TSLP promoter (–2056/–123 bp) linked to the luciferase gene was cloned by PCR from the human genomic DNA with the following primers: 5'-AGTGGTACCACTCTCTGGCCCTACAGCAA-3' and 5'-AGTAA GCTTCCACAGGAAGCCCTTATCA-3'. After PCR amplification, the fragment was digested with *Kpn*I/*Hind*III and then ligated into the pGL3.0 luciferase reporter vector (Promega, Madison, WI). Sequencing and insert orientation were verified by sequencing analysis.

Gene silencing

HIF-1 α siRNA (GenePharma, Shanghai, China) was used for HIF-1 α gene silencing, and scrambled siRNA was used as the control siRNA. At ~40% confluence, cells in 60-mm cell culture dishes were transfected with 20 nM siRNA using Lipofectamine (Invitrogen), according to the manufacturer's instructions. Cells were allowed to stabilize for 24 hours before being used in experiments.

Luciferase assay

HaCaT keratinocytes were seeded onto six-well plates and cotransfected with pGL3.0/TSLP-Luc plasmid with the control vector pRL-TK (Promega), using the Lipofectamine (Invitrogen). To correct variations in transfection efficiency, the Renilla luciferase encoding plasmid pRL-TK is commonly used for normalization controls. After 5 hours of transfection, cells were replaced with 0.5% fetal bovine serum DMEM for 16 hours and exposed to UVB. After UVB irradiation, the cells were incubated for 24 hours. In another condition, the transfected cells with pGL3.0/TSLP-Luc plasmid were cotransfected with the HIF-1 α expression vector or pcDNA3 vector. After incubation, the cells were collected and lysed with passive lysis buffer (Promega). Luciferase activity was determined using a dual-luciferase reporter assay system (Promega). Luminescence was measured using GloMax (Promega).

ChIP assay

HaCaT keratinocytes were irradiated with UVB and then incubated for 24 hours. Cells were treated with 1% formaldehyde for 10 minutes at 37°C. The cells were collected, and ChIP assay was performed using the ChIP assay kit from Upstate Biotechnology (Temecula, CA), according to the manufacturer's instructions. After immunoprecipitation, the captured genomic fragments were then recovered by phenol-chloroform extraction. Identification of the TSLP or VEGF promoter fragments was performed by PCR analysis, using promoter-specific primers. PCR was performed in 35 cycles, and the amplified products were analyzed on a 2% agarose gel. The antiserum used for the immunoprecipitation was directed against the HIF-1 α . The oligonucleotide sequences used for the PCR amplification of the TSLP and VEGF promoters were as follows: TSLP: forward, 5'-CCAGGTGACAGACGTTTCC-3', reverse, 5'-GGAGACGATGC TGTCTGAG-3' and forward, 5'-CCAAGGACCAGAGCGATG-3', reverse, 5'-CCTCTCTCTCTTCCCTTCC-3'; and VEGF: forward, 5'-GAGAGAGACGGGGTCAGAGAGAG-3', reverse, 5'-CAGCGCG ACTGGTCAGC TG-3'.

Immunohistochemistry

Paraffin tissue sections (4 μ m) were deparaffinized, rehydrated, and washed twice in buffer. To reduce nonspecific background staining due to endogenous peroxidase, the slides were incubated in hydrogen peroxide block for 10 minutes and washed four times in buffer. The primary antibody, anti-TSLP antibody (1:1000, antihuman TSLP polyclonal antibody; Abcam), was applied and incubated according to the manufacturer's recommended protocol, and the slides were washed four times in buffer. Then, primary antibody enhancer was applied to the slides, which were subsequently incubated for 20 minutes at room temperature and washed four times in buffer. Then, the horseradish peroxidase polymer was applied to the slides, and the slides were incubated for 30 minutes at room temperature and washed four times in buffer. They were then incubated with hematoxylin, washed four times in deionized water, and counterstained.

Statistics

All experiments were conducted in triplicate and independently repeated at least three times. The statistical significance of the data was determined by two-way analysis of variance using GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, CA). The results are expressed as mean $\pm$ SD. The level of significance was set at $P < 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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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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Supplementary Figure

Supplementary Figure S1.

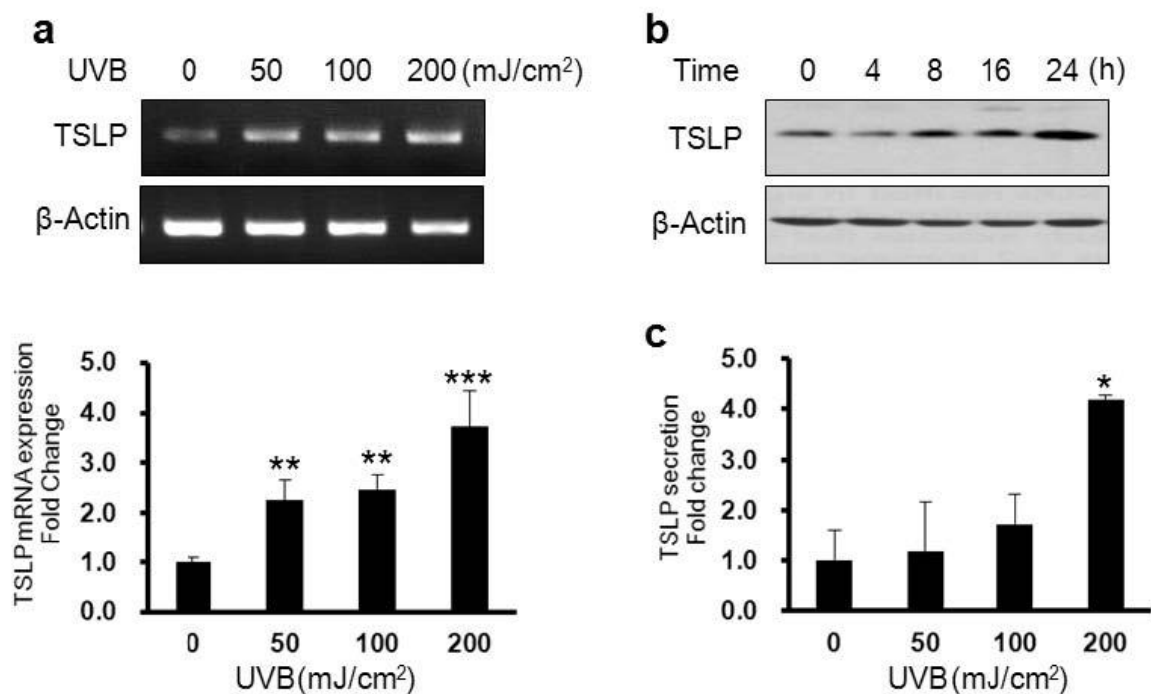


Figure S1. Upregulation of TSLP expression by UVB irradiation in NHEKs

(a) TSLP mRNA levels were upregulated by UVB irradiation in a dose-dependent manner. NHEKs were irradiated with different UVB doses (0, 50, 100, and 200 mJ/cm²) and then incubated for 24 hours. (b) Elevated TSLP protein expression was detected in a time-dependent manner. NHEKs were irradiated with 100 mJ/cm² of UVB and harvested at the indicated time points. Proteins were extracted from cells. (c) UVB irradiation increased TSLP protein secretion by NHEKs. Secreted TSLP levels in the supernatant of UVB-irradiated cells were measured by ELISA. TSLP levels were expressed in fold increase compared with the control. Asterisks indicate statistical significance at **p*<0.05, ***p*<0.01 and ****p*<0.001.