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Clinical and Experimental Dermatology

Promoter demethylation contributes to TSLP overexpression in skin lesions of patients with atopic dermatitis

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doi:10.1111/ced.12206

Summary

Background. Thymic stromal lymphopoietin (TSLP) plays an important role in promoting T-cell homeostasis, and appears to be a central player in the development of allergic symptoms, especially in asthma and atopic dermatitis (AD). Human TSLP is overexpressed in keratinocytes of patients with acute and chronic AD. However, the mechanism of TSLP overexpression remains unclear.

Aim. To investigate whether TSLP expression is regulated by aberrant DNA methylation modification of the TSLP promoter in keratinocytes of patients with AD.

Methods. mRNA and protein levels of TSLP in lesional skin samples from 10 children with AD and 10 healthy controls were measured by real-time quantitative reverse transcriptase-PCR and immunohistochemistry. Bisulfite sequencing was performed to determine the methylation status of the *TSLP* promoter, and 5-azacytidine (5-aza), a DNA methyltransferase inhibitor, was used to determine the influence of DNA methylation on *TSLP* expression.

Results. TSLP mRNA and protein expression levels were increased in skin lesions from patients with AD compared with healthy controls. Moreover, promoter hypomethylation of the *TSLP* gene was identified in skin lesions from patients with AD, and treating keratinocytes with 5-aza reduced the methylation level of the *TSLP* promoter and increased *TSLP* transcription.

Conclusions. DNA demethylation of a specific regulatory region of the *TSLP* gene may contribute to TSLP overexpression in skin lesions in patients with AD.

Introduction

Thymic stromal lymphopoietin (TSLP) was originally identified as a growth factor in conditioned medium of the Z210R.1 thymic stromal cell line.[1] In humans, TSLP, an interleukin (IL)-7-like cytokine molecule,[2] is produced mainly by human epithelial cells, including skin keratinocytes, tonsil-crypt epithelial cells and bronchial epithelial cells, and plays an important role in mediating allergic inflammation such as asthma and atopic dermatitis (AD).[3–5] The TSLP receptor (TSLPR) is mainly expressed on dendritic cells (DCs), and consists of a common IL-7 receptor-α (IL-7R-α) chain and a TSLP specific receptor-γ-like chain, TSLPR-γ.[6] TSLP activates DCs via TSLP–TSLPR interaction to promote innate immunity. Moreover, TSLP facilitates differentiation of naive CD4 + T cells into proinflammatory T helper (Th)2 cells.[7,8]

AD is a common skin disease characterized by dry skin, intense scratching and allergic inflammation of the dermis, with infiltration by T cells and accumulation of eosinophil-derived products.[9] Patients with AD have evidence of systemic Th2-dominant immune responses, and AD is often accompanied by other atopic disorders, such as asthma and allergic rhinitis.[10,11] Previous studies have shown that expression

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Conflict of interest: none declared.

Accepted for publication 16 March 2013

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of TSLP is significantly higher in keratinocytes of lesional skin from patients with acute and chronic AD.[3] However, the mechanisms regulating TSLP overexpression in AD remain unclear.

DNA methylation is known to regulate gene transcription. Methylation of the promoter or enhancer of target genes suppresses their transcription, whereas hypomethylation of the promoter or enhancer region is correlated with higher expression of those target genes.[12] Moreover, histone modifications are also involved in regulating configuration of chromatin and gene expression, thereby determining the accessibility and binding of activators and repressors of gene transcription.[13] Nakamura *et al.* reported that the DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) transcript is significantly repressed in peripheral blood mononuclear cells (PBMCs) of patients with AD who have high IgE levels,[14] suggesting that aberrant DNA methylation modification might be involved in the pathogenesis of AD. Recently, Liang *et al.* reported that DNA hypomethylation contributes to overexpression of the high-affinity IgE receptor FcεRI on monocytes of patients with AD.[15]

Therefore, we hypothesized that DNA hypomethylation leads to TSLP overexpression in skin lesions of patients with AD, and consequently, we investigated the putative role of DNA methylation in the regulation of TSLP expression in skin lesions of patients with AD. We also used 5-azacytidine (5-aza), a DNA methyltransferase inhibitor, to determine the influence of DNA methylation on TSLP expression in HaCaT cells.

Methods

The study was approved by the human ethics committee of Hunan Children's Hospital and the Second Xiangya Hospital of Central South University, and written informed consent was obtained from all subjects.

Subjects

In total, 10 patients with AD (mean ± SD age 9.1 ± 2.9 years, range 5–14) were recruited from the outpatient dermatology clinic and in-patient ward at Hunan Children's Hospital. The diagnosis of AD was confirmed using the Hanifin and Rajka diagnostic criteria.[16] Disease activity was assessed using the SCORing Atopic Dermatitis (SCORAD) index;[17] the SCORAD of patients with AD was > 25 (moderate or severe). Ten healthy children without AD (mean ± SD age 8.7± 1.6 years, range 7–12 years) were recruited from Hunan Children's Hospital as controls; these were matched for age and gender.

Skin-tissue samples

Samples of skin from the characteristic inflamed eczematous lesions from patients with AD were obtained. The healthy control samples for this experiment were obtained from non-atopic patients undergoing circumcision or cosmetic surgery.

Cell culture and treatment of 5-azacytidine

HaCaT cells were cultured in Dulbecco modified Eagle medium supplemented with 10% v/v fetal calf serum (Invitrogen Corp., Carlsbad, CA, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin (both Sigma-Aldrich, St Louis, MO, USA), at 37 °C in a 5% CO_2 humidified atmosphere. HaCaT cells were treated with 1 μmol/L 5-azacytidine (5-aza; Sigma-Aldrich) or solvent control (distilled water) in growth medium for 72 h as described previously.[18]

RNA isolation and real-time quantitative reverse transcriptase–PCR

Total RNA of skin tissues and HaCaT cells was isolated using Trizol reagent (Invitrogen Corp.). Real-time quantitative reverse transcriptase (qRT)-PCR was performed (Rotor-Gene 3000; Corbett Research, Mortlake, NSW, Australia) using the primers shown in Table 1, and mRNA levels were quantified (One Step PrimeScript RT-PCR Kit; Takara Bio Inc, Dalian, China). A dilution series of sample RNA was also

Table 1 Primers used in study.

	Direction	Sequence 5′→3′
Real-time qRT-PCR		
TSLP	Forward	GGGGCTAAACCATGACAGAA
	Reverse	ACCCTGAGCTTCTCTGGACA
β-actin	Forward	GCACCACACCTTCTACAATGAGC
	Reverse	GGATAGCACAGCCTGGATAGCAAC
Nested PCR		
Round 1		
−1488 to −1464	Forward	TTATGAAGAAGAGTTTAATTTTGGT
−1077 to −1053	Reverse	TCTATCTAACAATCTTCCCAATTCC
Round 2		
−1564 to −1540	Forward	TTTTAGTTTGTTTTTTATTTTTTTT
−1170 to −1146	Reverse	TACTCCTCCCTAACTTAACTCATAC

qRT-PCR, quantitative reverse transcriptase-PCR; TSLP, thymic stromal lymphopoietin

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included to generate a standard curve for calculation of relative concentrations of transcript in each RNA sample. β-actin was amplified and used as a loading control.

Genomic DNA extraction and bisulfite sequencing

Genomic DNA was isolated from skin tissues and HaCaT cells (TIANamp Genomic DNA kit; Tiangen Biotech, China). Genomic DNA bisulfite conversion was performed (EpiTect Bisulfite Kit; Qiagen Inc., Valencia, CA, USA). The 295 bp (–1465 to –1170 bp) *TSLP* promoter fragment was amplified by nested PCR, using the primers shown in Table 1. The PCR products were purified and cloned into the pGEM-T vector (Promega Corp., Madison, WI, USA), and then transformed into *Escherichia coli* cells. Ten positive recombinant clones were selected at random and sequenced (ABI 3730; Applied Biosystems). The methylation level of the gene was assessed according to the percentage of the methylated CG sites in the 10 clones.

Immunohistochemistry

The levels of TSLP protein in skin samples from healthy controls and from lesions from patients with AD was measured by immunohistochemistry (IHC) as described previously.[19] Two blinded observers counted anti-TSLP-labelled nuclei, and graded staining intensities (1 = mild, 2 = moderate or 3 = strong). Staining intensities were then evaluated semiquantitatively using the *H*-score equation:

$$\text{H-score} = \sum \alpha(x+1),$$

where x is the intensity grade (1, 2 or 3) and α is the percentage of nuclei stained with x intensity.

Western blotting

HaCaT cells were lysed, and protein was extracted and separated by 12% SDS-PAGE, then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). Membranes were blocked in Tris-buffered saline with 0.05% Tween 20 and 5% nonfat dry milk, then blotted with TSLP antibody (1: 200; Abcam, Cambridge, MA, USA) and β-actin antibody (1: 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed (Luminol/Enhancer Solution; Pierce Biotechnology Inc., Rockford, IL, USA). Experiments were repeated three times, and relative expression levels were quantified (Quantity One software; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Results are expressed as mean ± SD. Variables were compared using the independent samples *t*-test for samples of patients with AD and the paired-samples *t*-test for HaCaT cells with 5-aza treatment. $P < 0.05$ was considered significant. All results were analysed by SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA).

Results

Thymic stromal lymphopoietin is overexpressed in lesional skin from patients with atopic dermatitis

The mRNA expression level of TSLP relative to β-actin was significantly higher in AD lesional skin compared

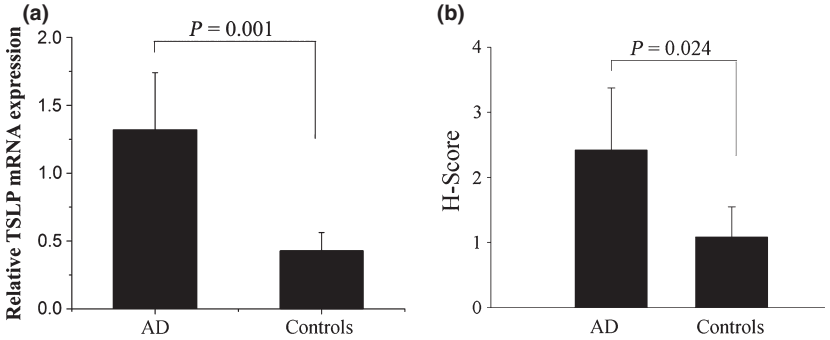

Figure 1 (a) Relative expression of thymic stromal lymphopoietin (TSLP) mRNA measured by quantitative real-time reverse-transcriptase-PCR. Results are presented as expression levels normalized to β-actin. TSLP mRNA expression was significantly increased in lesional skin from 10 patients with atopic dermatitis (AD) compared with 10 healthy controls ($P < 0.001$). (b) *H*-score for immunohistochemistry in the skin biopsies showed that TSLP protein levels were higher in the skin lesions from children with AD than in controls ($P = 0.02$).

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with control skin (1.32 ± 0.42 vs. 0.43 ± 0.14, respectively $P = 0.001$) (Fig. 1a). In addition, the IHC results showed upregulation of TSLP protein expression was in AD lesional skin compared with healthy control skin (*H*-score 2.43 ± 0.98 vs. 1.05 ± 0.66, $P = 0.02$) (Fig. 1b).

The thymic stromal lymphopoietin gene promoter is demethylated in lesional skin from patients with atopic dermatitis

The average methylation level of the *TSLP* promoter region containing 16 CG pairs was significantly lower in lesional skin from patients with AD compared with controls (34.38% ± 11.88% vs. 84.88% ± 7.51%, respectively, $P < 0.001$) (Fig. 2).

Inhibition of DNA methylation induces thymic stromal lymphopoietin overexpression in keratinocytes

TSLP mRNA level was higher in HaCaT cells treated with 5-aza than in the untreated negative controls (3.85 ± 0.30 vs. 0.51 ± 0.21, respectively, $P < 0.001$) (Fig. 3a) and TSLP protein level was also significantly increased in the 5-aza-treated HaCaT cells compared with untreated cells (0.76 ± 0.11 vs. 0.12 ± 0.10, $P < 0.001$) (Fig. 3b,c). The average methylation level of the *TSLP* promoter region containing 16 CG pairs was downregulated in the 5-aza-treated HaCaT cells compared with the negative controls (37.92% ± 13.49% vs. 85.83% ± 8.73%, $P < 0.001$) (Fig. 4).

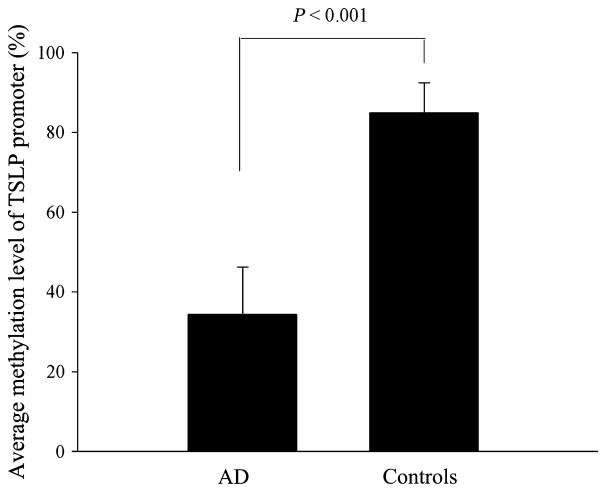

Figure 2 DNA was isolated from lesional skin of 10 patients with atopic dermatitis (AD) and 10 healthy controls, and DNA methylation level was measured by bisulfite sequencing to show thymic stromal lymphopoietin (TSLP) promoter methylation patterns. The average methylation level of *TSLP* promoter region containing 16 CG pairs was decreased in AD samples compared with control samples ($P < 0.001$).

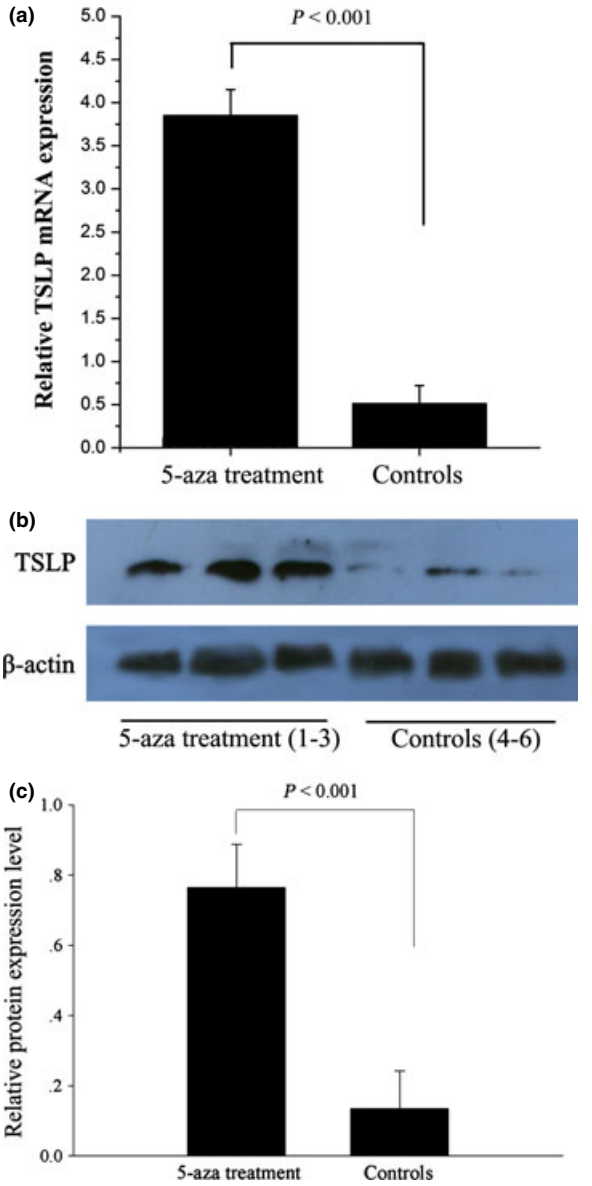

Figure 3 (a) Relative expression of thymic stromal lymphopoietin (TSLP) mRNA in HaCaT cells treated with 5-azacytidine (5-aza) or solvent control (distilled water), measured by quantitative real-time reverse-transcriptase-PCR. (b) TSLP protein levels in HaCaT cells treated with 5-aza or solvent control analysed by western blotting. (c) Band intensities from the western blots were calculated to measure the relative protein level of TSLP, and showed that TSLP mRNA and protein expression levels were significantly increased in 5-aza-treated HaCaT cells compared with solvent control cells ($P < 0.001$ for both mRNA and protein). Results are presented as expression levels normalized to β-actin, and represent the mean ± SD of three independent experiments per group.

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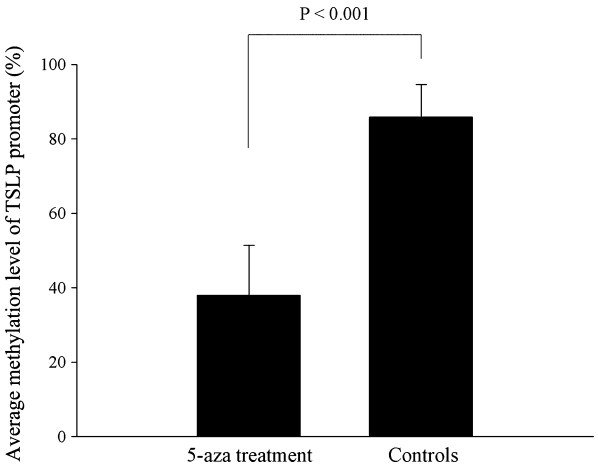

Figure 4 The change in thymic stromal lymphopoietin (TSLP) promoter methylation status in HaCaT cells treated with 5-azacytidine (5-aza) or solvent control (distilled water) was measured by calculating the average methylation level of the TSLP promoter region containing 16 CG pairs, and was found to be significantly reduced in 5-aza-treated HaCaT cells compared with solvent controls ($P < 0.001$). Data represent the mean ± SD of three independent experiments per group.

Discussion

AD is an inflammatory disorder characterized by infiltration and accumulation of memory-like Th2 cells and eosinophils.[20] Previous studies have shown that the TSLP protein is undetectable in normal skin, in skin lesions from patients with nickel-induced contact dermatitis or patients with disseminated lupus erythematosus, and in nonlesional skin from patients with AD; however, TSLP is overexpressed in both acute and chronic AD skin lesions.[3,4] Overexpression of TSLP by keratinocytes in the skin lesions of patients with AD can activate myeloid DCs by upregulating the surface expression on the myeloid DCs of CD54, CD80, CD83, CD86 and major histocompatibility complex class II molecules,[21] which results in a microenvironment permissive for Th2 inflammatory responses but not Th1-polarizing cytokines.[3] Although TSLP is implicated in the pathogenesis of AD, the mechanism underlying the overexpression of TSLP remains unclear. Aberrant DNA methylation plays an important role in various skin diseases.[22] For example, diesel exhaust causes hypermethylation of the interferon-γ gene promoter and hypomethylation of the IL-4 gene promoter, promoting induction of IgE in response to intranasal challenge with the allergen *Aspergillus fumigatus*.[23] Our previous studies have shown that promoter demethylation contributes to the upregulation of *FcεRIγ* transcription and surface expression of FcεRI in monocytes from patients with AD.[15] In this study, we found that both the mRNA and protein levels of TSLP were significantly increased in lesional skin from patients with AD, which is consistent with the previous report by other groups.[3] There was a concomitant pattern of *TSLP* promoter hypomethylation in skin lesions of patients with AD compared with healthy controls. Moreover, treating HaCaT cells with the demethylating agent 5-aza increased *TSLP* mRNA transcription amd reduced *TSLP* promoter methylation, which suggests that *TSLP* transcription may be regulated by DNA methylation. However, the mechanisms of *TSLP* demethylation still need to be investigated.

These various lines of evidence indicate that global hypomethylation of the genome accompanies the ageing process, concomitant with increased methylation of specific gene promoters,[24,25] suggesting epigenetic plasticity between early life adversity and epigenomic plasticity. According to this study, DNA hypomethylation contributes to overexpression of the *TSLP* gene in skin lesions from AD children. However, whether aberrant DNA methylation modification is also involved in regulating TSLP expression in adult patients with AD is unclear. In our previous work, we found that both global methylation and a locus-specific methylation at the *FCER1G* promoter were significantly reduced in monocytes from patients with AD aged 13–38 years, compared with healthy controls.[15] Another group reported that the levels of DNMT1 mRNA were significantly lower in PBMCs from patients with AD aged 16–47 years compared with normal controls.[14] These results suggest that DNA hypomethylation may also be involved in regulating TSLP expression in adult patients with AD, but further studies will be needed to confirm this hypothesis.

Conclusion

We found DNA hypomethylation and overexpression of TSLP in skin lesions from patients with AD, and confirmed that *TSLP* is a methylation-sensitive gene. Our findings confirm that DNA hypomethylation contributes to TSLP overexpression in AD skin lesions, suggesting that aberrant epigenetic modifications may play an important role in the pathogenesis of AD. These findings shed light on the role of aberrant epigenetic changes in the pathogenesis of AD, and indicate the possibility of epigenetic intervention in the treatment of AD. Further research is necessary to determine the causes leading to the dysregulation of epigenetic modifications in patients with AD.

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What's already known about this topic?

- Expression of TSLP is significantly higher in keratinocytes of lesional skin from people with acute and chronic AD.
- Aberrant DNA methylation modification has been identified in patients with AD.

What does this study add?

- DNA demethylation and overexpression of the *TSLP* gene were identified in skin lesions from patients with AD.
- 5-aza, a DNA methylation inhibitor, could induce TSLP overexpression in HaCaT cells.

Acknowledgements

This study was supported by National Basic Research Program of China (973 Plan) (no. 2009CB825605), the China Postdoctoral Science Foundation (grant no. 20100471235) and Hunan Postdoctoral Scientific Program (no. 2010RS4005).

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