Letter

An acid-hydrolyzed wheat protein activates the inflammatory and NF- κ B pathways leading to long TSLP transcription in human keratinocytes

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(Received August 19, 2019; Accepted March 16, 2020)

ABSTRACT — Hydrolyzed wheat proteins (HWPs) contained in cosmetics have occasionally caused immediate-type hypersensitivity following repeated skin exposure. Although the Cosmetic Ingredient Review Expert Panel concluded that < 3,500 Da HWP is safe for use in cosmetics, it remains biologically unknown how allergenic HWPs evoke immediate-type allergy percutaneously. Keratinocyte-derived thymic stromal lymphopoietin (TSLP) induces type 2 immune responses, which play an essential role in the pathogenesis of immediate-type allergy. Previously, we demonstrated that protein allergens in cultured human keratinocytes strongly induced long-form TSLP (loTSLP) transcription. However loTSLP-regulating signaling by HWP is poorly understood. In this study, we performed global gene expression analysis by microarray to investigate how the allergenic HWP acts on epidermal keratinocytes and the induction of loTSLP. Compared to human serum albumin (HSA), allergenic HWP induced a distinct gene expression pattern and preferentially activated various inflammatory pathways (High Mobility Group Box 1, Interleukin [IL]-6, IL-8, and acute phase response signaling). We identified 85 genes as potential nuclear factor-kappa B (NF- κ B) target genes in GP19S-treated cells, compared with 29 such genes in HSA-treated cells. In addition, HWP specifically altered IL-17 signaling pathways in which transcription factors, NF- κ B and activator protein-1, were activated. NF- κ B signaling may be an important factor for HWP-induced inflammatory loTSLP transcription via inhibition assay. In conclusion, allergenic HWP caused an easily sensitizable milieu of activated inflammatory pathways and induced NF- κ B-dependent loTSLP transcription in keratinocytes.

Key words: Hydrolyzed wheat protein, Keratinocytes, Thymic stromal lymphopoietin,
Nuclear factor-kappa B

INTRODUCTION

Hydrolyzed wheat proteins (HWPs) have been widely used in shampoos, conditioners, and moisturizers that are exposed to the skin. However, increasing evidence suggests that certain HWPs in cosmetics can cause contact urticaria or anaphylaxis (Niinimäki *et al.*, 1998; Varjonen *et al.*, 2000; Laurière *et al.*, 2006). In particular, acid-HWP, Glupearl 19S® (GP19S), caused an im-

mediate-type allergy outbreak which was associated with the use of facial soap in Japan (Fukutomi *et al.*, 2011; Yagami *et al.*, 2017). More recently Noguchi *et al.* (2019) reported that Japanese GP19S allergy strongly associated with HLA-DQ variants but not with FLG loss-of-function mutation in genome-wide association study. Conversely, several comparative studies among GP19S, other HWPs, and native gluten were conducted, and GP19S was found to contain novel epitopes and high molecular weight anti-

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gens (Nakamura *et al.*, 2013, 2016; Yokooji *et al.*, 2013). Our previous study, as well as others studies, demonstrated that compared with native gluten, GP19S possess higher allergenic potential than native gluten in mice and guinea pig models (Adachi *et al.*, 2012; Matsunaga *et al.*, 2015). In addition to host susceptibility, higher allergenicity of HWP itself may be vital for GP19S allergy. Although the Cosmetic Ingredient Review Expert Panel concluded that < 3,500 Da HWP was safe for use in cosmetics (Belsito *et al.*, 2014; Burnett *et al.*, 2018), it remains biologically unknown how allergenic HWPs, such as GP19S, evoke immediate-type allergy via the skin route.

Generally, an immediate-type allergy becomes sensitized by the antigen-uptake of antigen-presenting cells and antigen presentation to naïve T cells, leading to type 2 helper T (T_H2) cell expansion, development of T follicular helper cells, and IgE production. In the skin, an epithelial cell-derived cytokine, namely thymic stromal lymphopoietin (TSLP), acts as a type 2 immunity promoting factor. High TSLP levels were detected at the lesion sites of patients with atopic dermatitis and asthma, which mediated type 2 immunity (Soumelis *et al.*, 2002; Shikotra *et al.*, 2012). In addition, TSLP receptor signaling in Langerhans cells was essential for percutaneous sensitization with protein allergen and T_H2-type immune response in a murine model (Nakajima *et al.*, 2012). We recently reported that highly allergenic proteins, including GP19S, induced the long form of TSLP (loTSLP) in epidermal keratinocytes (Kuroda *et al.*, 2017). TSLP has two transcription variants. loTSLP plays a vital role in type 2 immune response-related allergic diseases, including atopic dermatitis, allergic rhinitis, and asthma. The short form of TSLP (shTSLP) is constitutively expressed at both mRNA and protein levels in the skin of healthy subjects and have anti-inflammatory property (Bjerkas *et al.*, 2015; Fornasa *et al.*, 2015). shTSLP is related to homeostatic functions in the thymus and gut (Tsilingiri *et al.*, 2017). Therefore, specific analysis of loTSLP is essential for detecting inflammatory responses. However the most studies have reported that the regulatory mechanisms of TSLP expression were analyzed as total-TSLP. Entire TSLP induction is regulated by NF-κB and activator protein 1 (AP-1) (Takai, 2012). In airway epithelial cells, IL-1β, and TNF-α-activated TSLP gene expression was promoted by the upstream NF-κB site (Lee and Ziegler, 2007). In the airway's smooth muscle cells, IgE-induced TSLP promoter activation was mediated by NF-κB and AP-1 (Redhu *et al.*, 2011). Furthermore, TSLP promoter region contained NF-κB, AP-1, STAT, and Smad binding sites in mice (Ganti *et al.*, 2017). Nev-

ertheless, little is known about the mechanisms of loTSLP transcription in epidermal keratinocytes. More recently, Redhu *et al.* (2020) reported four NF-κB binding sites were located in the human TSLP promoter region, and IL-1α promoted NF-κB recruitment to two active binding sites in human keratinocytes. In same setting, loTSLP mRNA was significantly augmented. Therefore, the binding NF-κB to these binding sites may important for loTSLP mRNA induction.

In this study, we performed comprehensive gene expression analysis to demonstrate the potential for biological predisposition to skin sensitization and induction of loTSLP by allergenic HWP. This study provides mechanistic insights into how highly allergenic proteins acts on epidermal keratinocytes.

MATERIALS AND METHODS

Cells

Primary human neonatal keratinocytes (Thermo Fisher Scientific/Life Technologies, Waltham, MA, USA) were cultured in HuMedia KG2 (Kurabo, Osaka, Japan) and seeded at 4.5×10^4 cells/well in flat-bottomed, 24-well culture plates. Once the cells reached 80% confluence (2-3 days after plating), the medium was replaced with HuMedia KG2, without hydrocortisone, in 24 hr according to Kinoshita *et al.* (2009)'s procedure. The cells that were passaged once were used for the assays.

Preparation of the exposure samples

GP19S, a product of wheat gluten obtained by partial hydrolysis with hydrogen chloride (HCl) at 95°C for 40 min, was supplied by Katayama Chemical, Inc. (Osaka, Japan). GP19S was suspended in 1 M tris (hydroxymethyl) aminomethane (Tris) solution (pH 11.4). After 24 hr at room temperature, the samples were neutralized with HCl by a previously reported method (Adachi *et al.*, 2012). A control vehicle sample (Tris-HCl) was prepared by neutralizing Tris with HCl. Human serum albumin (HSA; A5843, Sigma, St. Louis, MO, USA), with a confirmed endotoxin limit of 1.0 unit/mg, was dissolved in phosphate-buffered saline (PBS, pH 7.2; Wako Chemical, Osaka). Nuclear factor-kappa B (NF-κB) activation inhibitors, 4-methyl-N¹-(3-phenylpropyl)benzene-1,2-diamine (JSH-23; Merck KGaA, Darmstadt, Germany), and (1aR, 4E, 7aS, 10aS, 10bR)-2,3,6,7,7a,8,10a,10b-Octahydro-1a,5-dimethyl-8-methylene-oxireno[9,10] cyclodeca[1,2-b]furan-9(1aH)-one (Parthenolide; Sigma) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 3 and 10 μM, respectively. Another

NF- κ B activation inhibitor, ammonium pyrrolidine dithiocarbamate (PDTC; Sigma), was dissolved in PBS at a concentration of 100 μ M. The effectiveness of inhibitor concentrations was verified in the paper by Inman *et al.* (2008), Dommisch *et al.* (2010), and Galbiati *et al.* (2011).

Exposure, mRNA isolation, and cDNA synthesis

The inhibitors or vehicles were added to the cells an hour before sample exposure. Later, the cells placed in 400 μ L culture medium were treated with 100 μ L of the prepared sample for 3 hr. After removing the medium/test sample, the remaining adherent cells were processed for PCR analyses by the direct addition of 350 μ L of RLT buffer (Qiagen, Hilden, Germany) bearing 3.5 μ L of 2-mercapto-ethanol (Thermo Fisher Scientific) to each well. The total RNA was extracted from the lysed cells using the RNeasy Micro Kit in combination with QIAshredder (Qiagen) according to the manufacturer's instructions. After confirming RNA concentration and purity using the NanoDrop 2000 System (Thermo Fisher Scientific), the first-strand of cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). These experiments were conducted in triplicate for statistical analysis.

Microarray and pathway analysis

Total RNA quality and quantity was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop 2000. An Affymetrix GeneChip Human Gene 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) was used for the microarray experiment according to the manufacturer's instructions. GeneSpring GX 14.5 (Agilent Technologies) was used to filter and normalize the raw data. RMA16 and a baseline transformation to the medians of all samples were used. For statistical analysis, one-way ANOVA was applied for multiple group comparison, followed by multiple testing correction and setting of the false discovery rate to 0.05 using the Benjamini and Hochberg method. Differentially expressed genes (DEGs) were determined using this statistical analysis and fold change cut-off of 2.0 for GP19S vs. Tris-HCl and HSA vs. PBS. A list of DEGs was uploaded to the Ingenuity Pathway Analysis (IPA, Qiagen) software to investigate the biological networks resulting from the altered global gene expression. IPA generated the activation score (Z-score) and the p-value for canonical pathways and upstream regulator analysis.

Real-time quantitative PCR analysis

Real-time PCR analysis was performed using the TaqMan® Universal PCR Master Mix and Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The total-TSLP (toTSLP) and β -actin transcription levels were analyzed using PCR primer sets recommended by Thermo Fisher Scientific (Hs00263639_m1 and Hs01060665_g1). The loTSLP transcription was analyzed using a specific long form probe set (Hs01572933_m1). The primer and probe sets for shTSLP were designed and synthesized by TaqMan MGB probe service (Applied Biosystems) from the shTSLP-specific region of human TSLP, transcript variant 2, mRNA (NM_138551.4), according to Kuroda *et al.* (2017). The relative gene expression levels were normalized to β -actin, and the differences, when compared to control CT values, were calculated using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Unpaired student's t-test (2-tailed) was used for statistical analyses. $p < 0.05$ indicated statistical significance.

RESULTS

GP19S produced a unique expression pattern when compared with HSA and solvents

According to our previous work, GP19S, unlike HSA, induced potent loTSLP transcription (Supplementary Fig. 1). To elucidate this difference, the gene expression profiles of keratinocytes exposed to 3 mg/mL GP19S and 66 mg/mL HSA were compared. The microarray data were deposited into Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>), with accession number GSE138725. A total of 513 DEGs were identified between GP19S-exposed condition and the solvent control, including 404 upregulated and 109 downregulated genes. On the other hand, 187 DEGs between HSA-exposed condition and solvent control, including 117 upregulated and 70 downregulated genes, were identified (Fig. 1A). GP19S induced 2.7-times more DEGs than HSA. GP19S induced 396 non-overlapped DEGs (Fig. 1B) and completely different expression patterns when compared to HSA and solvent control (Fig. 1C).

GP19S preferentially induced inflammation-related pathways

To investigate which canonical pathways were preferentially activated in GP19S-exposed keratinocytes, the expression profiles of 513 and 187 DEGs that were altered in GP19S and HSA-treated keratinocytes, respectively, were applied to pathway analyses. The inflammation-related pathways, such as High Mobility Group Box

Fig. 1. Comparison of DEGs between GP19S-treated and HSA-treated epidermal keratinocytes. (A) The numbers of upregulated and downregulated DEGs in the GP19S-treated and HSA-treated epidermal keratinocytes were determined using statistical analysis and a 2.0 fold change cut-off for GP19S vs Tris-HCl and HSA vs PBS. (B) A Venn diagram depicts the overlap of DEGs in the GP19S-treated and HSA-treated epidermal keratinocytes. (C) A heat map depicting hierarchical clustering of DEGs in the GP19S-treated, HSA-treated, and solvent-treated samples (green = upregulation, red=downregulation).

1 (HMGB1), Interleukin (IL)-6, IL-8, and acute phase response signaling, were identified as preferentially GP19S-activated (Fig. 2). A more detailed analysis of the components of these pathways revealed that c-Fos, nuclear factor-kappa B (NF- κ B), and the target genes (IL-6, Tumor Necrosis Factor [TNF]- α , and IL-1) were upregulated (Tables 1 and 2). Moreover, upstream analysis used to predict the regulators confirmed more GP19S activated putative inflammatory regulators than HSA (Fig. 3A). For instance, upstream analysis identified 85 potential NF- κ B target genes in GP19S-treated cells when compared with 29 such genes in HSA-treated cells (Fig. 3B, Supplementary Table 1).

GP19S specifically induced NF- κ B, AP-1, and C/EBP β

To clarify the influence of GP19S but not HSA, the

specifically induced factors of 396 DEGs, which were altered only in GP19S-treated keratinocytes, were applied to the canonical pathway analyses. Several IL-17 related pathways were identified (Fig. 4). A more detailed investigation of the IL-17 signaling pathway components revealed that the components of NF- κ B, AP-1, and CCAAT/enhancer binding protein (C/EBP β) were upregulated (Table 3).

Inhibition of NF- κ B activation abrogates I α TSLP transcription

From the above results, NF- κ B signaling appeared to be an important factor serving as a GP19S-preferred and GP19S-specific activator. Therefore, we investigated whether inducing I α TSLP mRNA by GP19S was influenced by 3 NF- κ B activation inhibitors, namely JSH-23 (selective blocker of the nuclear translocation of RelA),

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Table 1. DEGs within the genes involved in the HMGB1 signaling pathway.

Gene symbol	Gene description	GP19S Fold Change	HSA Fold Change
CCL2	C-C motif chemokine ligand 2	2.1	-
CSF2	colony stimulating factor 2	3.3	-
CXCL8	C-X-C motif chemokine ligand 8	36.7	4.8
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	10.6	-
ICAM1	intercellular adhesion molecule 1	2.3	-
IL11	interleukin 11	2.2	-
IL1A	interleukin 1 alpha	3.4	3.1
IL1B	interleukin 1 beta	3.4	2.6
IL6	interleukin 6	33.8	-
IRS2	insulin receptor substrate 2	-	2.2
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	12.2	2.1
LIF	LIF, interleukin 6 family cytokine	8.2	6.8
NFKB1	nuclear factor-kappa B subunit 1	3.7	-
NFKB2	nuclear factor-kappa B subunit 2	2.2	-
RHOB	ras homolog family member B	2.8	-
RND3	Rho family GTPase 3	4.3	2.1
SERPINE1	serpin family E member 1	3.1	-
TGFB2	transforming growth factor beta 2	2.2	-
TNF	tumor necrosis factor	8.3	-

Fig. 2. Comparison analysis of canonical signaling pathways activated by GP19S and HSA treatment. A list of top 10 canonical pathways of DEGs in GP19S or HSA-treated samples is shown with Z-scores.

parthenolide (inhibitors of I κ B α degradation and RelA nuclear translocation), and PDTC (antioxidant). As the keratinocytes were pretreated with each NF- κ B activation inhibitor, loTSLP mRNA induction by sufficient amounts of GP19S (100 and 500 μ g/mL) was almost completely blocked (Fig. 5). In contrast, shTSLP mRNA induction by 100 μ g/mL GP19S was not significantly blocked, and shTSLP mRNA induction by 500 μ g/mL GP19S was significantly blocked, but not as much as loTSLP mRNA.

Fig. 3. Upstream regulator analysis of DEGs in epidermal keratinocytes after GP19S and HSA treatments. (A) A list of top 11 upstream regulators of DEGs in GP19S or HSA-treated samples is shown with Z-scores. (B) The numbers of putative downstream factors regulated by NF- κ B (complex) in the GP19S-treated and HSA-treated epidermal keratinocytes.

The cytotoxic effects of all NF- κ B activation inhibitors were not observed at the tested concentration (data not shown). The obtained data suggest that NF- κ B activation plays pivotal roles in loTSLP transcription and partial shTSLP transcription induced by protein allergens.

Table 2. DEGs within the genes involved in the IL-6 signaling pathway.

Gene symbol	Gene description	GP19S Fold Change	HSA Fold Change
CEBPB	CCAAT enhancer binding protein beta	2.1	-
CXCL8	C-X-C motif chemokine ligand 8	36.7	4.8
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	10.6	-
IL1A	interleukin 1 alpha	3.4	3.1
IL1B	interleukin 1 beta	3.4	2.6
IL1RL1	interleukin 1 receptor like 1	2.4	-
IL1RN	interleukin 1 receptor antagonist	2.6	-
IL36G	interleukin 36 gamma	2.2	3.3
IL6	interleukin 6	33.8	-
IRS2	insulin receptor substrate 2	-	2.2
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	12.2	2.1
NFKB1	nuclear factor-kappa B subunit 1	3.7	-
NFKB2	nuclear factor-kappa B subunit 2	2.2	-
NFKBIA	NFKB inhibitor alpha	10.8	-
NFKBID	NFKB inhibitor delta	2.2	-
SOCS3	suppressor of cytokine signaling 3	6.6	-
TNF	tumor necrosis factor	8.3	-

Fig. 4. Canonical pathway analysis of GP19S-specific DEGs in epidermal keratinocytes. A list of top 17 canonical pathways of DEGs, specifically affected by GP19S, are shown with p-values. Orange squares indicated the ratio of the number of DEGs that map to a given pathway divided by the total number of genes that are involved in the pathway.

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Fig. 5. Impact of NF- κ B activation inhibitors on short and long TSLP transcription induced by GP19S. Short and long TSLP transcription, induced by 100 and 500 μ g/mL GP19S, was affected by NF- κ B activation inhibitors (JSH-23, Parthenolide, and PDTC) in epidermal keratinocytes. * $p < 0.01$ t-test vs. DMSO + GP19S group.

DISCUSSION

For preventing allergic outbreaks, it is important to elucidate the underlying mechanisms of percutaneous sensitization by highly allergenic HWPs. This study revealed the highly allergenic HWP, GP19S, induced a distinct gene expression pattern and preferentially various inflammatory pathways compared to HSA in epidermal keratinocytes. In these pathways, NF- κ B, AP-1, and C/EBP β were activated. In addition, NF- κ B signaling seems to be an important factor in HWP-induced inflammatory loTSLP transcription.

It was reported that the ability of food allergens, such as ovalbumin, to pass through the skin to the draining lymph node was quite limited and did not result in specific IgE development when the protein was applied on intact skin (Dioszeghy *et al.*, 2011; Li *et al.*, 2012). However, percutaneous application of the macromolecular allergenic HWP, dissolved in aqueous buffer, induced systemic type 2 immune response and immediate-type hypersensitivity in guinea pigs (Matsunaga *et al.*, 2015) and mice (Adachi *et al.*, 2012). These findings imply that external proteins usually minimally penetrate into the epidermis and are therefore insufficient to cause sen-

sitization; nonetheless, allergic HWP activates the epidermal cells and results in epidermal immune cell activation and/or enhanced skin permeability. In epidermal keratinocytes, highly allergenic HWP induced 2.7-times as many DEGs than the low allergenic HSA. In pathway analyses, the HWP-specific DEGs were linked to various inflammatory cytokines (IL-6, IL-1, and TNF) that stimulate dendritic cells (Toebak *et al.*, 2009). HWP-activated keratinocytes may provide an environmental niche for efficient sensitization. We previously reported that IL-17 impaired the tight junction barrier (Yuki *et al.*, 2016). Although IL-17 was basically supplied by the T cells and did not exist in our experimental system, other HWP-activated signaling pathways may cross talk with IL-17 signaling and be relevant to skin permeability. In the case of anaphylaxis caused by HWP-containing facial soap in Japan, surfactants may have enhanced the sensitivity of the inflammatory response to HWP. In the preliminary data ($n = 2$), the pathway analysis of DEGs (fold change > 2) altered in 0.5 mg/mL wheat gluten-treated keratinocytes showed that IL-6 and some inflammatory signaling pathways were detected in the top 10 canonical pathways (Fig. 6A), whereas that altered in 68 mg/mL ovalbumin (low endotoxin)-treated keratinocytes showed that inflam-

Fig. 6. Canonical pathway analysis activated by wheat gluten and ovalbumin treatment. The lists of top 10 canonical pathways of DEGs affected by wheat gluten (A) or ovalbumin (B) are shown with p-values. Orange squares indicated the ratio of the number of DEGs that map to a given pathway divided by the total number of genes that are involved in the pathway.

matory signaling pathways were not preferentially activated (Fig. 6B). Because wheat gluten, but not ovalbumin, can be sensitized by application to intact skin, the findings of HWP in this study will expand to other protein allergens which can be sensitized via the skin route.

In this study, among the various HWP materials, we focused on allergenic GP19S, which was acid-hydrolyzed for a short duration (40 min). Our research, as well as other works, has demonstrated that GP19S stimulated anaphylactic response by percutaneous sensitization in animal models and induced inflammatory signaling and NF- κ B-dependent lTSLP transcription in epidermal keratinocytes (Matsunaga *et al.*, 2015, Kuroda *et al.*, 2017). Such potentials of other HWP mate-

rials, which were hydrolyzed by acid, alkali, or enzymatic treatment, are largely unknown. Wheat gluten, when acid-hydrolyzed for 9 hr, induced slightly weaker anaphylactic reaction in guinea pig model than the compound acid-hydrolyzed for merely 0.5 hr (Matsunaga *et al.*, 2015). Moreover, when wheat gluten was acid-hydrolyzed for 6 hr, it induced lower lTSLP transcription and TSLP protein release in keratinocytes than the one acid-hydrolyzed for 0.5 hr (Kuroda *et al.*, 2017). Since the wheat gluten acid-hydrolyzed for 9 hr had a lower molecular weight than the one acid-hydrolyzed for 0.5 hr, we predicted that small HWPs do not induce inflammatory signaling and NF- κ B-dependent lTSLP transcription in keratinocytes. However, further work is needed to investigate whether

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Table 3. GP19S-specific DEGs within the genes involved in the IL-17A signaling pathway.

Gene symbol	Gene description	Fold Change
CCL2	C-C motif chemokine ligand 2	2.1
CEBPB	CCAAT enhancer binding protein beta	2.1
CEBPD	CCAAT enhancer binding protein delta	2.4
CXCL5	C-X-C motif chemokine ligand 5	6.6
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	10.6
IL6	interleukin 6	33.8
NFKB1	nuclear factor-kappa B subunit 1	3.7
NFKB2	nuclear factor-kappa B subunit 2	2.2
NFKBIA	NFKB inhibitor alpha	10.8
NFKBID	NFKB inhibitor delta	2.2

Table 4. A list of top 10 upstream regulators of DEGs in wheat gluten-treated epidermal keratinocytes.

Upstream Regulator	Activation z-score	p-value
IL1B	5.62	6.95E-36
TNF	6.43	2.17E-33
Pkc(s)	3.36	2.06E-26
PDGF BB	4.94	1.17E-23
EGF	4.25	8.3E-20
RELA	4.11	9.16E-20
HGF	3.61	1.18E-19
NfκB (complex)	5.04	2.06E-19
Vegf	4.95	2.81E-19
P38 MAPK	3.05	4E-19

Table 5. A list of top 10 upstream regulators of DEGs in ovalbumin-treated epidermal keratinocytes.

Upstream Regulator	Activation z-score	p-value
TNF	5.16	7.52E-31
IL1B	4.26	1.5E-30
IL1A	3.76	9.67E-27
DUSP1	-2.58	7.21E-22
IL17A	3.26	1.77E-21
NFKBIA	1.00	4.23E-21
CSF2	3.74	1.49E-20
PDGF BB	3.40	3.79E-20
NfκB (complex)	3.96	7.5E-20
ZFP36	-2.80	1.08E-19

other safely used and allergenic HWPs induce inflammatory signaling and NF-κB-dependent loTSLP transcription in epidermal keratinocytes.

We proved that HWP activated NF-κB, AP-1, and C/EBPβ. Harada *et al.* (2009) reported that poly(I:C)-induced loTSLP transcription was regulated by the AP-1 binding site in human bronchial epithelial cells. More recently, Li *et al.* (2018) proposed that human metapneumovirus infection specifically induced loTSLP transcription, which depended on NF-κB activation in human airway epithelial cell lines. Despite the cell source differences, these findings are consistent with our results that allergenic HWPs induced loTSLP mRNA via NF-κB signaling pathway. In our preliminary data (n = 2), upstream regulator analysis estimated that wheat gluten and ovalbumin, which are known loTSLP inducers, significantly activated NF-κB (Tables 4 and 5). Two NF-κB activation inhibitors (JSH-23 and parthenolide) blocked the nuclear translocation of RelA (p65). Vu *et al.* (2011) demonstrated that RelA small interfering RNA transfection inhibited poly(I:C)-induced TSLP mRNA augmentation in epidermal keratinocytes. Apart from us, other researchers also established that poly(I:C) could induce loTSLP mRNA

(Xie *et al.*, 2012; Kuroda *et al.*, 2017). Therefore, RelA is a pivotal factor for inducing loTSLP transcription. In our system, two blockers of the nuclear translocation of RelA (JSH-23 and parthenolide) inhibited loTSLP mRNA upregulation by HWP. We conclude that NF-κB, especially RelA, strictly associated with HWP-inducing loTSLP transcription. Although AP-1 can influence loTSLP induction by protein allergens, further study is needed to clarify its role.

Further, little is known regarding shTSLP transcription. Tsilingiri *et al.* (2017) mentioned that shTSLP was upregulated by Vitamin D in epithelial cells and in silico analysis revealed that Vitamin D receptor binding site was identified in the upstream of the short isoform open reading frame. Martin Mena *et al.* (2017) reported that shTSLP mRNA expression was decreased by the peroxisome proliferator activated receptor-gamma knock-down or this antagonist in epithelial cells. To our knowledge, this is the first study to demonstrate that NF-κB may regulate the inducible shTSLP transcription. ShTSLP exon is shared with loTSLP exon, although each promoter was different. Therefore, shTSLP transcription may be influenced by loTSLP-regulating factors like NF-κB.

On the other hand, shTSLP mRNA expression was not changed when NF- κ B was recruited to binding sites of human TSLP promoter by IL-1 α in human keratinocytes (Redhu *et al.*, 2020). In our inhibition assay, the inhibitory effects of NF- κ B inhibitors on shTSLP upregulation was much weaker than that on loTSLP induction. Accordingly, NF- κ B components without RelA may need for shTSLP induction.

In conclusion, an allergenic HWP induced an easily sensitizable milieu by activating several inflammatory pathways and induced NF- κ B-dependent loTSLP transcription in epidermal keratinocytes. Comprehensive identification of inflammatory pathways and loTSLP transcriptional data can help estimate the allergen's epidermal sensitizing potency as well as the sensitizing mechanisms of type 2 immune response and immediate-type hypersensitivity.

Conflict of interest---- Kayoko Matsunaga is the principle investigator of the laboratory funded by Hoya Co., Ltd. and received research fund from Yuuka Co., Ltd.. These funders had no role in the study design, conduct of the study, data collection, data interpretation or preparation of the manuscript.

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