Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes

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Background: Atopic dermatitis (AD) is a chronic inflammatory skin disorder caused by multiple factors. Among them, house dust mite (HDM) allergens are important in the development of AD. In airway allergy, HDM allergens activate innate immunity. However, information regarding the activation of innate immunity by HDM allergens in the skin is limited.

Objectives: The inflammasome is a key regulator of pathogen recognition and inflammation. We investigated whether HDM allergens activate the inflammasome in epidermal keratinocytes.

Methods: Keratinocytes were stimulated with *Dermatophagoides pteronyssinus*, and the activation of caspase-1 and secretion of IL-1β and IL-18 were examined. Formation of the inflammasome was studied by analyzing the subcellular distributions of inflammasome proteins. The importance of specific inflammasome proteins was studied by knocking down their expression through transfection of keratinocytes with lentiviral particles carrying short hairpin RNAs (shRNAs).

Results: *D pteronyssinus* activated caspase-1 and induced caspase-1–dependent release of IL-1β and IL-18 from keratinocytes. Moreover, *D pteronyssinus* stimulated assembly of the inflammasome by recruiting apoptosis-associated specklike protein containing a caspase-recruitment domain (ASC), caspase-1, and nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain containing 3 (NLRP3) to the perinuclear region. Finally, infection with lentiviral particles carrying ASC, caspase-1, or NLRP3 shRNAs suppressed the release of IL-1β and IL-18 from the keratinocytes. Activation of the NLRP3 inflammasome by *D pteronyssinus* was dependent on cysteine protease activity.

Conclusion: House dust mite allergens are danger signals for the skin. In addition, HDM-induced activation of the NLRP3 inflammasome may play a pivotal role in the pathogenesis of AD. (J Allergy Clin Immunol 2011;127:806-14.)

Key words: *Keratinocytes, house dust mite, caspase-1, IL-1β, IL-18, NLRP3, inflammasome,* Dermatophagoides pteronyssinus*, Der p 1*

Abbreviations used

AD:	Atopic dermatitis
ASC:	Apoptosis-associated specklike protein containing a caspase-recruitment domain
E-64:	Trans-epoxysuccinyl L-leucylamido(4-guanidine)butane
HDM:	House dust mite
HMGB1:	High-mobility group box protein 1
NF-κB:	Nuclear factor-κB
NLR:	Nucleotide-binding oligomerization domain–like receptor
NLRP:	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin-domain containing
PAR2:	Protease-activated receptor 2
PMA:	Phorbol 12-myristate 13-acetate
PolyI:C:	Polyinosinic-polycytidylic acid
shRNAs:	Short hairpin RNAs
TLR:	Toll-like receptor
zVAD:	Benzyloxycarbonyl-valine-alanine-aspartate
zYVAD:	Benzyloxycarbonyl-tyrosine-valine-alanine-aspartate

Atopic dermatitis (AD) is a chronic inflammatory skin disorder affecting 10% to 20% of children worldwide.[1,2] It sometimes persists into adulthood and has a significant impact on the quality of life of patients and their families. Its incidence has increased over recent decades.[1] Its pathogenesis involves interactions among multiple factors including susceptibility genes, environmental factors, skin barrier defects, and immunologic factors.[2,3]

Among environmental factors, house dust mite (HDM) allergens are important for the development of AD as well as asthma and rhinitis.[4-6] *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the most common types of HDM in temperate climates and are known to contribute to the immunopathogenesis of AD through the induction of IgE binding.[7] In airway allergy, activation of innate immunity by HDM allergens plays an important role in disease pathogenesis.[6,8] However, little is known about the activation of innate immunity by HDM allergens in the skin.

The innate immune system senses invading pathogens via evolutionarily conserved pathogen-recognition receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain–like receptors (NLRs),[9,10] which play a central role in both innate immunity and inflammatory diseases.[10-12] NLR members form a multiprotein complex, the inflammasome, which activates caspase-1 and ultimately leads to the processing and release of the proinflammatory cytokines IL-1β, IL-18, and IL-33.[12]

There is growing evidence that inflammasomes play important roles in skin inflammation.[13] Among them, much attention has focused on the nucleotide-binding oligomerization domain,

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leucine-rich repeat and pyrin domain containing 3 (NLRP3) (also known as NACHT, LRR, and PYD domains-containing protein 3 [NALP3] or cryopyrin) inflammasome, which is made up of NLRP3, apoptosis-associated specklike protein containing a caspase-recruitment domain (ASC), and caspase-1.[12] Autoinflammatory diseases are characterized by recurrent episodes of fever and skin rash caused by excessive release of IL-1β, which is a result of active mutations in *NLRP3* and can be alleviated by IL-1 receptor antagonists.[14] In addition, the NLRP3 inflammasome is a key regulator of contact hypersensitivity.[13] A recent report linked the NLRP3 inflammasome to allergic diseases,[15] revealing associations between 2 *NLRP3* single nucleotide polymorphisms that increase the expression and activity of NLRP3 and susceptibility to food-induced anaphylaxis and aspirin-induced asthma. Elsewhere, it has been shown that the majority of patients with AD are colonized by *Staphylococcus aureus*,[16] and that hemolysins and bacterial lipoproteins from *S aureus* can activate the NLRP3 inflammasome.[17,18]

The inflammasome can be activated not only by pathogen-associated molecules but also by various other stimuli, including danger-associated molecular pattern molecules such as ATP and urate crystals, environmental stimuli such as silica crystals and aluminum salts,[19] and neurodegenerative stimuli such as amyloid-β fibrils.[20]

In this study, we investigated whether HDM allergens activate the keratinocyte inflammasome. We present evidence that HDM allergens activate the NLRP3 inflammasome and stimulate keratinocytes to release the proinflammatory cytokines IL-1β and IL-18. The release of these cytokines may trigger or exacerbate AD-associated inflammation.

METHODS

Cell culture

Primary human keratinocytes were isolated from neonatal skin samples discarded after surgery. This study was conducted according to the principles of the Declaration of Helsinki, and all procedures involving human subjects received previous approval from the ethics committee at the Ehime University School of Medicine, Japan. Written consent was provided by patient guardians before experiments were initiated. Keratinocytes were cultured in MCDB153 medium (Nissui, Tokyo, Japan) as described previously.[21]

Reagents and cell stimulation

Phorbol 12-myristate 13-acetate (PMA) and polyinosinic-polycytidylic acid (polyI:C) were obtained from Invivogen (San Diego, Calif). Standardized lyophilized extracts of HDM *D pteronyssinus* and *D farinae* were obtained from COSMO BIO (Tokyo, Japan). Affinity chromatography–purified natural allergens (Der p 1 and Der f 1) and the recombinant allergen Der p 2 were obtained from Indoor Biotechnologies (Cardiff, United Kingdom).

Keratinocytes were stimulated with allergens suspended in serum-free medium. Trans-epoxysuccinyl L-leucylamido(4-guanidine)butane (E-64; Sigma-Aldrich, St Louis, Mo) was used as a specific cysteine protease inhibitor.[22,23] Before being added to the cultures, E-64 was incubated at 37°C for 15 minutes in medium containing allergens. To inactivate allergen proteases, HDM extract was incubated at 65°C for 30 minutes.[22] In a subset of experiments, the pan-caspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate (zVAD) and the caspase-1–specific inhibitor benzyloxycarbonyl-tyrosine-valine-alanine-aspartate (zYVAD; Enzo Life Sciences, Plymouth Meeting, Pa) were added to cultures before the application of allergens.

RNA preparation and real-time RT-PCR

Total RNA was isolated by using Isogen (Nippon Gene, Tokyo, Japan). Real-time RT-PCR was performed by using an ABI PRISM 7700 sequence detector (Applied Biosystems, NJ). Primers and probes specific for GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), IL-1β, and IL-8 were obtained from Applied Biosystems. PCR analysis was performed by using a TaqMan RT-PCR Master Mix Reagent Kit (Applied Biosystems) according to the manufacturer's protocol. Target gene expression was normalized to the GAPDH signal. Levels of gene expression in allergen-treated cells were quantified relative to those in untreated cells.

ELISA

After the incubation of cells with allergens, cell culture supernatants were collected and stored at –20°C. The release of IL-1β, IL-8, IL-1α and caspase-1 was quantified by using ELISA kits from R&D Systems (Minneapolis, Minn) and that of IL-18 by using an ELISA kit from MBL (Nagoya, Japan).

Protein isolation and Western blotting

After stimulation of cells, cell lysates and supernatants were separated by SDS-PAGE and analyzed by using a Vistra ECF Kit (GE, Tokyo, Japan). Membranes were scanned by using a FluoroImager (Molecular Dynamics, Sunnyvale, Calif). The following primary antibodies were used: rabbit anti-IκBα (inhibitor of kappa B-alpha), anti–phospho-IκBα, anti–caspase-1, and anti–IL-1β (Cell Signaling, Danvers, Mass)[24]; rabbit anti–IL-18 (MBL); rabbit anti-ASC; mouse anti-NLRP1 and anti-NLRP3 (Abnova, Walnut, Calif); rabbit anti-high-mobility group box protein 1 (HMGB1) (Abcam, Cambridge, Mass) and rabbit anti-actin (Santa Cruz Biotechnology, Santa Cruz, Calif).

Immunofluorescence microscopy

Keratinocytes were fixed with 4% paraformaldehyde and incubated overnight at 4°C with antibodies raised against caspase-1, ASC, and NLRP3. They were then treated with an Alexa Fluor 488–conjugated secondary antibody and DAPI (Invitrogen, Carlsbad, Calif). The cells were washed with PBS and mounted by using VECTASHIED (Vector, Burlingame, Calif). Images were acquired by using a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Lentiviral transfection

Keratinocytes were transfected with lentiviral particles (at a multiplicity of infection of 0.4) carrying control, ASC, caspase-1, or NLRP3 shRNAs (Santa Cruz Biotechnology) in the presence of 4 μg/mL polybrene (Santa Cruz Biotechnology), according to the manufacturer's instructions. Knockdown of ASC, caspase-1, and NLRP3 was verified in pooled cell populations by nested RT-PCR and Western blotting.

Statistical analysis

For each analysis, at least 3 independent experiments were performed, all of which yielded similar results. Data from representative experiments are shown. Relative mRNA expression and levels of secreted cytokines are presented as the means ± SDs ($n > 3$). Statistical significance was determined by using Student *t* tests. A P value $<.05$ was considered statistically significant.

RESULTS

HDM extracts induce caspase-dependent secretion of IL-1β and IL-8 from keratinocytes

Because nuclear factor-κB (NF-κB) is a central mediator of inflammatory responses, we first studied whether HDM allergens activate NF-κB signaling and regulate cytokine expression in keratinocytes. PMA and polyI:C were used as positive controls.[25] Whereas PMA and polyI:C triggered rapid phosphorylation of IκBα, *D pteronyssinus* and *D farinae* did not (Fig 1, *A*). Furthermore, they did not influence IL-1β mRNA levels. In contrast, PMA and polyI:C increased IL-1β mRNA levels as soon as 3 hours after stimulation (Fig 1, *B*). Although *D pteronyssinus*