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*

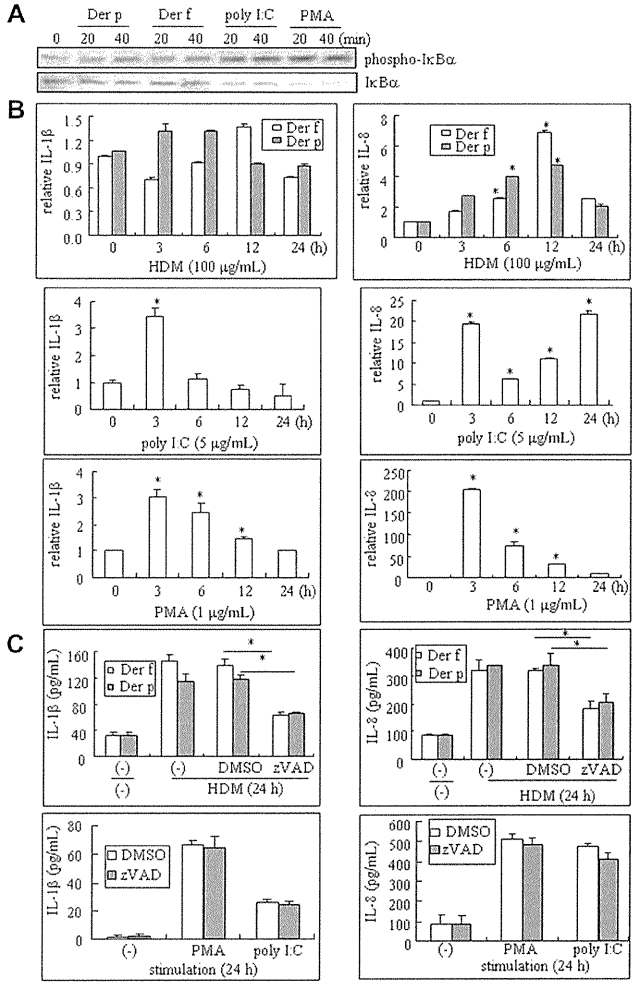

FIG 1. HDM extracts induce caspase-dependent secretion of IL-1β and IL-8. Keratinocytes were treated with *D pteronyssinus (Der p), D farinae (Der f),* PMA and polyI:C. **A,** Cellular IκBα and phospho-IκBα levels were detected by Western blotting. **B,** Levels of IL-1β and IL-8 mRNA were measured by real-time RT-PCR. Relative mRNA levels are presented as the means ± SDs (n = 5); $*P < .05$. **C,** Keratinocytes were stimulated with HDM extracts, PMA, and polyI:C in the presence or absence of zVAD (10 μmol/L). Levels of secreted IL-1β and IL-8 were measured by ELISA. Concentrations are presented as the means ± SDs (n = 4); $*P < .05$. *DMSO,* Dimethyl sulfoxide.

and *D farinae* elicited approximately 4-fold and 7-fold increases, respectively, in IL-8 mRNA expression, with peak induction occurring 12 hours after stimulation (Fig 1, *B*), their effects were modest and gradual compared with those of polyI:C and PMA.

We next analyzed the release of IL-1β and IL-8 from cultured keratinocytes. Although HDM did not alter keratinocyte expression of IL-1β mRNA, it significantly enhanced the release of IL-1β and IL-8 from cultured cells (Fig 1, *C*), as did PMA and polyI:C (Fig 1, *C*).

Pretreatment of keratinocytes with the pan-caspase inhibitor zVAD reduced HDM-induced IL-1β release and IL-8 release by approximately 60% and 40%, respectively (Fig 1, *C*), but did not modulate the responses to PMA and polyI:C. Thus, HDM allergens stimulate keratinocyte production of IL-1β and IL-8 in a caspase-dependent manner, despite their inability to activate NF-κB signaling.

Because keratinocytes respond similarly to *D farinae* and *D pteronyssinus*, henceforth we present data obtained by using only *D pteronyssinus*.

D pteronyssinus triggers the activation of caspase-1 and cleavage of pro–IL-1β and pro–IL-18 in keratinocytes

Although *D pteronyssinus* did not enhance the expression of pro–IL-1β mRNA (Fig 1, *A*), primary keratinocytes expressed detectable amounts of pro–IL-1β (Fig 2, *A, upper*), the likely source of mature IL-1β. In parallel with a gradual decrease in intracellular pro–IL-1β levels after *D pteronyssinus* stimulation, supernatant levels of mature IL-1β increased in a time-dependent fashion, as detected by Western blotting (Fig 2, *A, upper*), with cleaved p17 transiently detected in cellular lysates (Fig 2, *A, upper*). In contrast, the supernatant pro–IL-1β levels were not increased after stimulation with *D pteronyssinus* (Fig 2, *B, upper*). These data suggest that the time-dependent secretion of IL-1β from *D pteronyssinus*–treated keratinocytes, as detected by ELISA (Fig 2, *B, lower*), largely relates to mature IL-1β and not inactive pro–IL-1β. Like IL-1β, the cytosolic precursor of IL-18 was converted to its biologically active form in HDM-treated keratinocytes. After *D pteronyssinus* stimulation, active, mature IL-18 was rapidly secreted, as shown by Western blotting and ELISA analyses of cell culture supernatants, with intracellular levels of pro–IL-18 decreasing in parallel (Fig 2, *B*).

Caspases are proteases with regulatory roles in inflammation and apoptosis. There are 11 known caspases in human beings, but only caspase-1 has been shown to mediate the processing of pro–IL-1β and pro–IL-18.[26] We investigated the role of caspase-1 in HDM-induced activation of IL-1β. Caspase-1 is initially expressed as an inactive precursor that, after stimulation, is cleaved, yielding active p10 and p20 subunits.[27] Intracellular active p20 was detected as soon as 3 hours after *D pteronyssinus* stimulation (Fig 2, *C, upper*). ELISA analysis of supernatants from *D pteronyssinus*–treated keratinocytes further revealed time-dependent release of active caspase-1 p10 and p20 (Fig 2, *C, lower*). Thus, *D pteronyssinus* stimulates the activation of caspase-1 in keratinocytes. The kinetics of caspase-1 activation, which was initially detected 3 hours after stimulation, and the activation of IL-1β and IL-18, which increased subsequently, indicate the dependence of cytokine release on caspase-1.

Concomitant exposure to the caspase-1–specific inhibitor zYVAD blocked the activation of caspase-1 (Fig 2, *D*) and significantly inhibited the processing and secretion of IL-1β (Fig 2, *E*) and IL-18 (Fig 2, *F*). Thus, *D pteronyssinus* stimulates caspase-1 activation, which is necessary for the activation and secretion of IL-1β and IL-18 from keratinocytes.

D pteronyssinus activates the NLRP3 inflammasome in keratinocytes

Primary keratinocytes stably expressed the inflammasome proteins caspase-1 (Fig 2, *A*), ASC (Fig 3, *A*), and NLRP3 (Fig 3, *A*), but not NLRP1 (data not shown). Keratinocyte expression of ASC and NLRP3 was upregulated by treatment with *D pteronyssinus* (Fig 3, *A*). Caspase-1 is activated as a result of inflammasome assembly, which occurs in the cytosol.[28] To investigate whether *D pteronyssinus* triggers the formation of inflammasomes in keratinocytes, we examined the subcellular localization of inflammasome proteins by immunofluorescence.

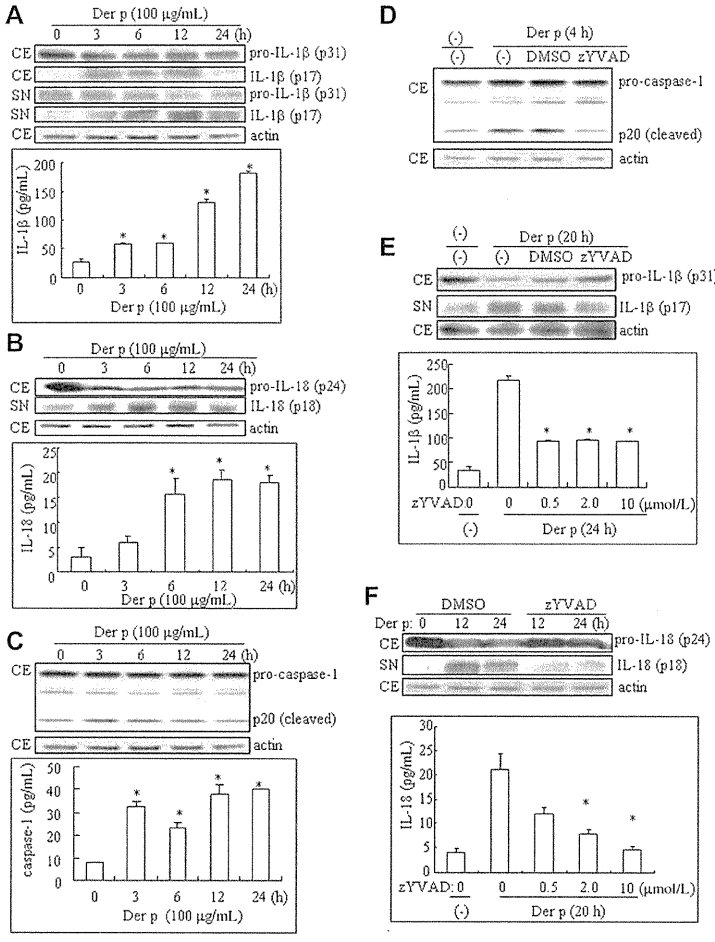

FIG 2. *D pteronyssinus (Der p)* induces caspase-1–dependent secretion of IL-1β and IL-18. Keratinocytes were treated with Der p for the indicated periods. **A,** Levels of pro–IL-1β and active mature IL-1β in cell lysates and supernatants. **B,** Levels of pro–IL-18 (intracellular) and active mature IL-18 (in supernatants). **C,** Levels of active caspase-1 in cell lysates (as measured by Western blotting) and supernatants (by ELISA). Keratinocytes were incubated with Der p in the presence of zYVAD (10 μmol/L), and cellular caspase-1 levels **(D)** and supernatant IL-1β levels **(E)** were measured by Western blotting. The effects of different doses of zYVAD on IL-1β release were assessed by ELISA *(E)* and the effects of zYVAD on the activation and release of IL-18 by Western blotting and ELISA **(F).** Concentrations are presented as the means ± SDs (n = 4); $*P < .05$. *CE,* Cell extracts; DMSO, Dimethyl sulfoxide; *SN,* supernatants.

ASC is sequestered in the nucleus of resting phagocytic cells and only becomes available for the bridging of NLRs and caspase-1 in the cytosol.[28] In resting keratinocytes, ASC predominantly localized to the nucleus, as determined by nuclear counterstaining (Fig 3, *B, left*). However, treatment with *D pteronyssinus* stimulated the rapid redistribution of ASC to the cytosol. At 4 hours posttreatment, ASC was detected throughout activated cells but was predominantly localized to the perinuclear region (Fig 3, *B, left*). Caspase-1 staining showed diffuse expression in the cytosol of resting cells and perinuclear aggregation in *D pteronyssinus*–treated cells (Fig 3, *B, middle*). Specific staining for NLRP3 revealed its diffuse distribution throughout resting cells. After stimulation, NLRP3 rapidly translocated to the cytosol and accumulated in the perinuclear region (Fig 3, *B, right*). Thus, in keratinocytes, ASC, caspase-1, and NLRP3 accumulate in the perinuclear region in response to *D pteronyssinus* stimulation, suggesting that *D pteronyssinus* activates NLRP3 inflammasomes in keratinocytes.

To test whether inflammasome proteins are involved in caspase-1/IL-1β activation, we infected keratinocytes with lentiviral particles carrying ASC, caspase-1, and NLRP3 shRNAs, which efficiently knocked down protein expression, as assessed by Western blotting (Fig 3, *C*). After transfection, supernatant levels of active caspase-1/IL-1β were measured. *D pteronyssinus* treatment significantly increased supernatant levels of active caspase-1 (Fig 3, *D*) and secreted IL-1β (Fig 3, *E*) and IL-18 (Fig 3, *F*). These responses were greatly attenuated in cells expressing ASC, caspase-1, and NLRP3 shRNAs, but not in others expressing a control shRNA. Thus, ASC, caspase-1, and NLRP3 are apparently all essential for the release of IL-1β and IL-18.

To test whether K^+ efflux is required for *D pteronyssinus*–induced activation of the NLRP3 inflammasome, we stimulated keratinocytes with *D pteronyssinus* in the presence of KCl (1-100 mmol/L) or identical concentrations of NaCl (control). The activation and release of caspase-1 (Fig 4, *A*), IL-1β (Fig 4, *B*), and IL-18 (Fig 4, *C*) in response to treatment with *D*

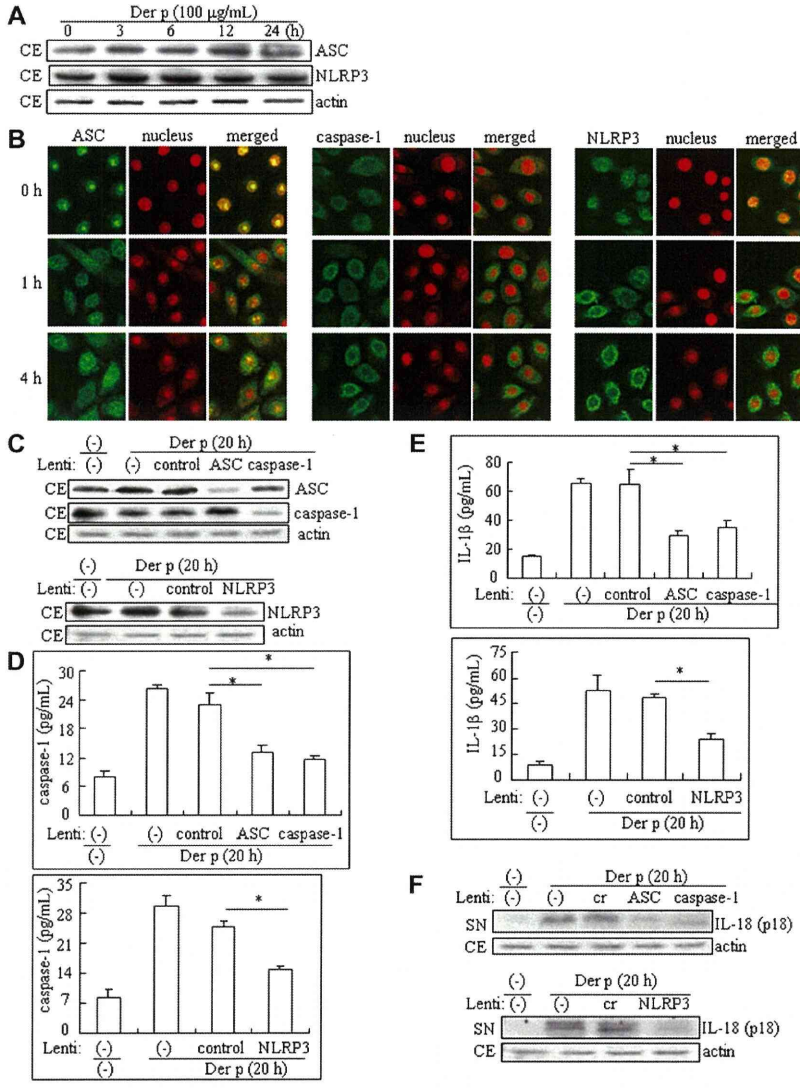

FIG 3. *D pteronyssinus (Der p)* activates the NLRP3 inflammasome in keratinocytes. **A,** Levels of cellular ASC and NLRP3 were estimated by Western blotting. **B,** Subcellular distribution of ASC, caspase-1, and NLRP3 in keratinocytes treated with Der p (for 0, 1, and 4 hours), assessed by immunofluorescence. Keratinocytes were infected with lentiviral particles *(Lenti)* carrying ASC, caspase-1, NLRP3, and control *(cr)* shRNAs, and then treated with Der p. Levels of ASC, caspase-1, and NLRP3 were determined by Western blotting **(C),** and the release of caspase-1 **(D),** IL-1β **(E),** and IL-18 **(F)** was assessed by ELISA or Western blotting. Concentrations are presented as the means ± SDs (n = 4); $*P < .05$. *CE,* Cell extracts; *SN,* supernatants.

pteronyssinus was blocked when the extracellular KCl concentration was increased to 100 mmol/L. Therefore, *D pteronyssinus*–mediated activation of the NLRP3 inflammasome depends on the efflux of K^+ ions.

Thus, *D pteronyssinus* activates the NLRP3 inflammasome in keratinocytes, which is in turn required for the activation of caspase-1 and release of IL-1β and IL-18.

D pteronyssinus–induced caspase-1/IL-1β activation depends on cysteine protease activity

We next investigated whether HDM proteases are involved in inflammasome activation. Heat treatment of *D pteronyssinus*, which inactivates its proteolytic activities,[22,29] almost completely blocked IL-1β release (Fig 5, *A*), suggesting that proteolytic activity is indeed required for NLRP3 inflammasome–mediated IL-1β secretion. We further tested the effects of specific protease inhibitors. Preincubation of *D pteronyssinus* with the cysteine protease inhibitor E-64 not only impaired the activation of caspase-1 (Fig 5, *B*) but also inhibited the release of IL-1β (by approximately 60%; Fig 5, *C*) and IL-18 (Fig 5, *D*). In contrast, the specific serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride[23] did not block the release of IL-1β (data not shown). These data indicate that the activation of the NLRP3 inflammasome by *D pteronyssinus* was, at least in part, dependent on HDM-derived cysteine protease activity.

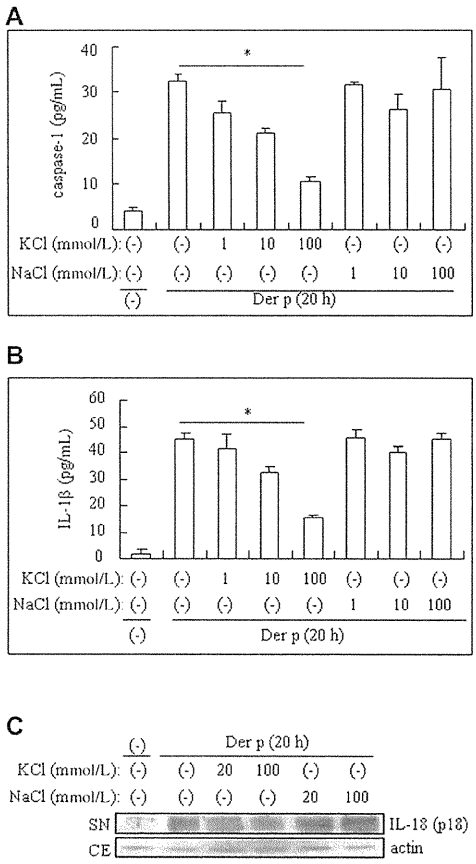

FIG 4. Extracellular K^+ inhibits *D pteronyssinus (Der p)*–induced activation of the NLRP3 inflammasome. Keratinocytes were treated with Der p for 20 hours in the presence of KCl or NaCl, and supernatant levels of active caspase-1 **(A)**, IL-1β **(B)**, and IL-18 **(C)** were measured. Concentrations are presented as the means ± SDs (n = 4); *$P < .05$. *CE,* Cell extracts; *SN,* supernatants.

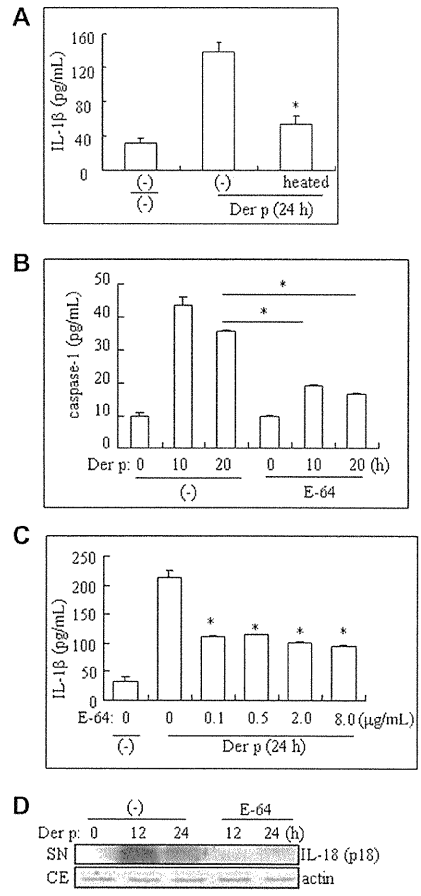

FIG 5. Cysteine protease activity is required for *D pteronyssinus (Der p)*–mediated inflammasome activation. **A,** Der p was heat-treated and then applied to cultures. IL-1β release was measured. Der p was or was not preincubated with E-64 (2 μg/mL) before being added to cultures. Supernatant levels of active caspase-1 **(B)**, IL-1β **(C)**, and IL-18 **(D)** were subsequently measured. Concentrations are presented as the means ± SDs (n = 4); *$P < .05$. *CE,* Cell extracts; *SN,* supernatants.

Der p 1 activates caspase-1 and induces the release of IL-1β and IL-18

Because cysteine protease activity is essential for *D pteronyssinus*–mediated inflammasome activation (Fig 5), we investigated whether the group 1 allergens, which exhibit cysteine protease activity,[23,30] were capable of activating the inflammasome. Purified natural Der p 1 caused significant activation of caspase-1 (Fig 6, *A*) and the release of considerable amounts of IL-1β (Fig 6, *B*) and IL-18 (Fig 6, *C*). These responses were nearly fully abrogated by the preincubation of Der p 1 with E-64. Furthermore, we showed, by using zYVAD, that the Der p 1–induced release of IL-1β and IL-18 from keratinocytes depended on caspase-1 activity (Fig 6, *D and E*). Stimulation with Der f 1 yielded similar results (data not shown). These data indicate that group 1 allergens stimulate inflammasome activation and induce the release of IL-1β and IL-18, responses that depend on their cysteine protease activity.

DISCUSSION

The current study provides the first evidence that HDM allergens trigger assembly of the NLRP3 inflammasome, activate caspase-1, and thus stimulate the processing and release of IL-1β and IL-18. The secretion of IL-1β usually requires two separate signals. The first, from a pathogen-associated molecular pattern molecule, promotes intracellular production of the immature cytokine. The second, derived from a danger signal, triggers inflammasome assembly, caspase-1 activation, and secretion of mature IL-1β.[26] However, the IL-1β and IL-18 released from *D pteronyssinus*–treated keratinocytes appear to originate from prestored pools of precursor cytokines, because no priming of immature cytokine production was detected. The response to *D pteronyssinus* bears some similarities to necrosis-induced NLRP3 activation, in which inflammasome activation and the release of caspase-1 do not depend on LPS priming or other proinflammatory stimuli.[31] However, cell necrosis was not observed in our cultures.

K^+ efflux seems to be the best common and specific trigger of NLRP inflammasome activation,[32] including the *D pteronyssinus*–mediated activation of the NLRP3 inflammasome in keratinocytes. Other important intracellular compounds include reactive oxygen species, which are often required for activation of the NLRP3 inflammasome.[33] However, preincubation of cells with the broad-spectrum NADPH oxidase inhibitor diphenyleneiodonium failed to impair *D pteronyssinus*–induced IL-1β

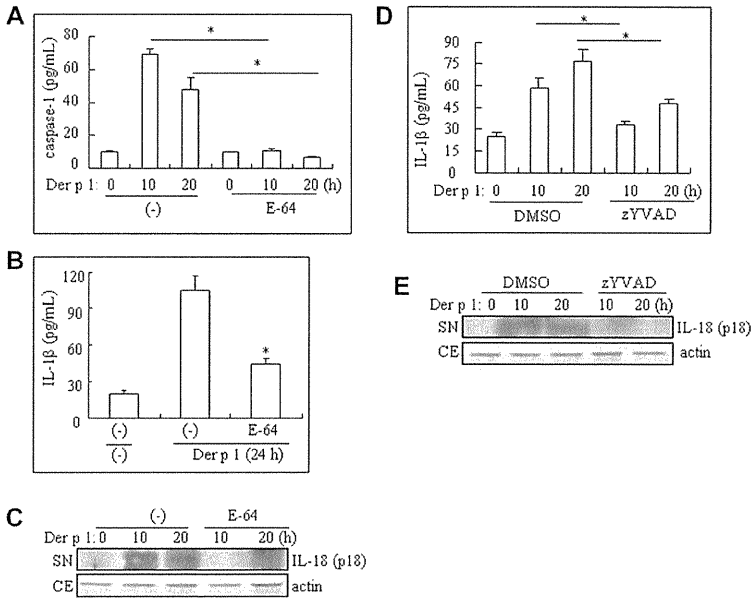

FIG 6. Der p 1 activates the inflammasome in keratinocytes. Keratinocytes were treated with Der p 1 (10 μg/mL) preincubated or not preincubated with E-64 (2 μg/mL), and the release of active caspase-1 **(A)**, IL-1β **(B)**, and IL-18 **(C)** was assessed. Cells were treated with Der p 1 in the presence or absence of zYVAD (10 μmol/L). Supernatant levels of mature IL-1β **(D)** and IL-18 **(E)** were subsequently measured. Concentrations are presented as the means ± SDs (n = 4); $*P < .05$. *CE,* Cell extracts; *DMSO,* Dimethyl sulfoxide; *SN,* supernatants.

activation (data not shown), suggesting that *D pteronyssinus* activates the NLRP3 inflammasome independently of reactive oxygen species. ATP is an activator of inflammasomes[34] and may mediate *D pteronyssinus*–induced inflammasome activation in keratinocytes. However, extracellular ATP failed to trigger the release of IL-1β from *D pteronyssinus*–nontreated[35] or *D pteronyssinus*–treated keratinocytes (data not shown), suggesting that ATP is not involved in activation of the inflammasome by *D pteronyssinus*, at least in keratinocytes.

High-mobility group box protein 1 (HMGB1) is a nuclear protein that acts as a powerful proinflammatory cytokine when released from cells.[36] According to a recent report, HMGB1 activates the inflammasome in dendritic cells.[37] We performed western blotting of supernatants and detected the release of HMGB1 from *D pteronyssinus*–treated keratinocytes (see this article's Fig E1 in the Online Repository at www.jacionline.org). Thus, it is possible that HMGB1 is involved in *D pteronyssinus*–mediated inflammasome activation in keratinocytes. Further studies are needed to confirm whether danger molecules such as HMGB1 are involved in *D pteronyssinus*–mediated activation of the NLRP3 inflammasome.

Skin barrier dysfunction is one of the major manifestations of AD and a key contributor to its pathogenesis.[3] Barrier disturbances resulting from genetic defects,[38,39] abnormal expression of epidermal proteins caused by T_H2-type cytokines,[40,41] or protease allergens[42,43] may increase the risk of sensitization to allergens and contribute to the development of AD. However, how physical defects result in immunologic abnormalities remains unclear. Barrier defects may allow environmental allergens, such as HDM allergens, to penetrate the underlying tissues and increase the likelihood of epidermal keratinocytes being exposed to them.

Although Gutgesell et al[44] reported that HDM elimination strategies do not improve disease activity in patients with AD, other studies have described the beneficial effects of HDM avoidance in limiting eruptions.[45,46] We suggest that HDM allergens contribute to the development of AD by activating the NLRP3 inflammasome and triggering the release of the proinflammatory cytokines IL-1β and IL-18. Although most clinical features of AD are a direct consequence of T_H2-skewed acquired immune responses,[16] patients with AD appear to develop a biphasic T_H-cell pattern, characterized by T_H2 cytokine production early during the acute phase, followed by a switch to a more T_H1-like profile during the chronic phase. IL-1β and IL-18, both members of the IL-1 family,[47] are important contributors to T_H1 responses[48] and also regulate T_H2 responses.[49] IL-18 has been shown to regulate allergic inflammation by inducing T_H2 cytokine production and eosinophilia.[50,51] HDM-induced release of IL-18 from keratinocytes may, therefore, be important in the pathogenesis of AD.

Recent studies using mouse models have further indicated the importance of IL-1β and IL-18 for the development of AD. Yamanaka et al[52] demonstrated that skin-specific caspase-1–transgenic mice maintained under specific pathogen-free conditions spontaneously develop chronic dermatitis, accompanied by abnormally elevated skin and serum IL-18 and IL-1β levels. Elsewhere, an *Il18*-transgenic mouse that exhibited oversecretion of IL-18 from epidermal cells developed AD-like skin eruptions, which improved after deletion of the *Il18* gene.[53] Of potential interest is the fact that the skin phenotypes of *Il18*-transgenic mice closely resemble those of *Nlrp3*-knockin mice. Furthermore, in an intrinsic AD mouse model[54] generated through the daily application of protein A (an *S aureus* surface molecule and virulence factor), administrating a neutralizing anti–IL-18 antibody and knocking out the *Il18* gene completely ameliorated the AD-like skin eruptions.

In addition to its effects on IL-1β and IL-18, *D pteronyssinus* stimulated the release of IL-1α (an unconventional leadless

protein but not a protease substrate[55]), at least partially in a caspase-1–dependent manner (see this article's Fig E2 in the Online Repository at www.jacionline.org). Thus, HDM allergen-mediated activation of the NLRP3 inflammasome and subsequent release of IL-1 proteins may be important in the development of dermatitis.

Mite cysteine proteases (group 1 allergens) and serine proteases (group 3, 6, and 9 allergens) both stimulate cells, but by different mechanisms. Der p 3 and Der p 9 induce the release of IL-8 and GM-CSF from keratinocytes[23] and airway epithelial cells[56,57] by activating protease-activated receptor 2 (PAR2) signaling. In contrast, Der p 1 and Der f 1, instead of activating PAR2 signaling, stimulate the inflammasome in keratinocytes.[23,56] Because epidermal keratinocytes and airway epithelial cells respond to HDM allergens in similar ways,[23,56,57] it is possible that *D pteronyssinus* and Der p 1 may trigger activation of the NLRP3 inflammasome in airway epithelial cells. This should be studied further.

The group 2 allergens are major antigens for IgE and lack protease activity.[58] Unlike Der p 1, Der p 2 did not stimulate the release of IL-1β from keratinocytes but did stimulate IL-8 release (see this article's Fig E3 in the Online Repository at www.jacionline.org) via a nonproteolytic mechanism, presumably involving the TLR2/myeloid differentiation primary response gene 88 (MyD88)–dependent activation of NF-κB and mitogen-activated protein kinase signaling pathways.[59,60] Thus, keratinocytes are able to sense HDM allergens directly and to initiate local inflammatory responses, perhaps through the cooperation of PAR2, inflammasomes, and even TLR pathways.

In conclusion, HDM allergens are novel, effective activators of the inflammasome in epidermal keratinocytes. Notably, HDM allergen-mediated activation of the NLRP3 inflammasome, and consequent release of IL-1 family proteins, may be important in the development of atopic dermatitis.

Clinical implications: Although mite allergens have previously been shown to activate TLRs, this is the first study to show that mite allergens directly activate the inflammasome in epidermal keratinocytes.

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METHODS

Cell cultures

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 as described previously.[E1]

Reagents and cell stimulation

Standardized lyophilized extract of HDM *D pteronyssinus* was obtained from LSL (Japan), and the recombinant allergen Der p 2 was obtained from Indoor Biotechnologies Ltd (Cardiff, United Kingdom). Keratinocytes were stimulated with allergens suspended in serum-free medium. To inhibit the activation of caspase-1, the specific caspase-1 inhibitor zYVAD (Alexis, Pa) was added to cultures before the application of allergens.

ELISA

After incubating cells with allergens, cell culture supernatants were collected and stored at –20°C. The release of IL-1α, IL-1β, and IL-8 was quantified by using commercially available ELISA kits (R&D Systems).

Protein isolation and Western blotting

After stimulation of cells for the indicated times, cell lysates and supernatants were separated by SDS-PAGE and analyzed by using a Vistra ECF Kit (Amersham Biosciences, Ill), and membranes were scanned by using a FluoroImager (Molecular Dynamics, Calif). The antibodies used were HMGB1 from Abcam and antiactin from Santa Cruz Biotechnology.

Statistical analysis

For each analysis, at least 3 independent experiments were performed, all of which yielded similar results. Data from representative experiments are shown. Statistical significance was determined by using Student *t* tests. A P value <.05 was considered statistically significant.

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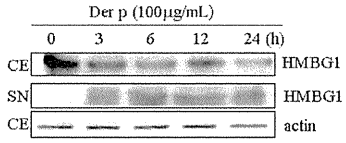

FIG E1. *D pteronyssinus (Der p)* stimulates the release of HMGB1 from keratinocytes. Keratinocytes were treated with Der p (100 μg/mL) for the indicated times. The levels of intracellular and supernatant HMGB1 were assessed by Western blotting. *CE,* Cell extracts; *SN,* supernatants.

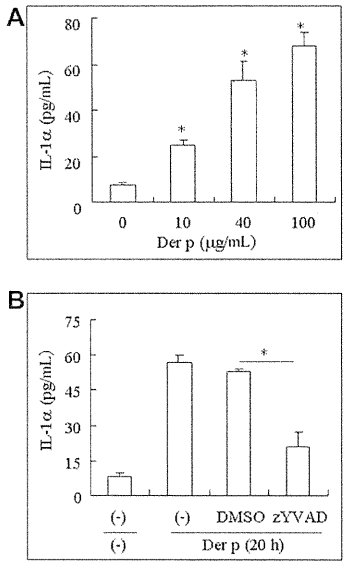

FIG E2. *D pteronyssinus (Der p)* induces caspase-1–dependent secretion of IL-1α. **A,** Keratinocytes were stimulated with increasing concentrations of Der p for 20 hours. The release of IL-1α was detected by ELISA. **B,** Keratinocytes were incubated with Der p (100 μg/mL) in the presence or absence of zYVAD (10 μmol/L). The supernatant levels of IL-1α were measured by ELISA. Concentrations represent means ± SDs (n = 4); **P* < .05. *DMSO,* Dimethyl sulfoxide.

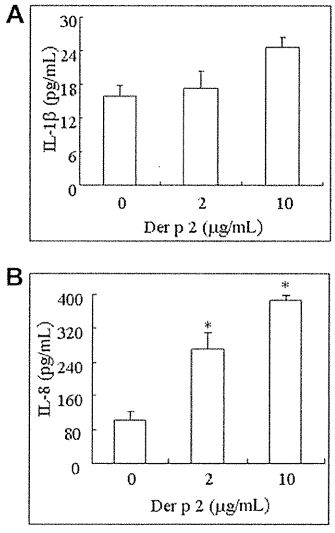

FIG E3. Der p 2 does not stimulate the release of IL-1β from keratinocytes. Keratinocytes were treated with increasing concentrations of Der p 2 for 24 hours, and the secretion of IL-1β **(A)** and IL-8 **(B)** was detected by ELISA. Concentrations represent means ± SDs (n = 4); $*P < .05$.

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Nuclear translocation of phosphorylated STAT3 regulates VEGF-A-induced lymphatic endothelial cell migration and tube formation

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific growth factor that regulates endothelial functions, and signal transducers and activators of transcription (STATs) are known to be important during VEGF receptor signaling. The aim of this study was to determine whether STAT3 regulates VEGF-induced lymphatic endothelial cell (LEC) migration and tube formation. VEGF-A (33 ng/ml) enhanced LEC migration by 2-fold and increased tube length by 25% compared with the control, as analyzed using a Boyden chamber and Matrigel assay, respectively. Western blot analysis and immunostaining revealed that VEGF-A induced the nuclear translocation of phosphorylated STAT3 in LECs, and this translocation was blocked by the transfection of LECs with an adenovirus vector expressing a dominant-negative mutant of STAT3 (Ax-STAT3F). Transfection with Ax-STAT3F also almost completely inhibited VEGF-A-induced LEC migration and tube formation. These results indicate that STAT3 is essential for VEGF-A-induced LEC migration and tube formation and that STAT3 regulates LEC functions.

1. Introduction

Blood vessels are essential for supplying oxygen and nutrients to tissues and for disposing of metabolic waste products, in order to maintain optimal tissue homeostasis and function. Lymphatic vessels drain protein-rich lymph from the extracellular space to maintain normal tissue pressure and are important for trafficking immune cells to the lymph nodes. Both blood vessels and lymphatic vessels are central to cancer progression and metastasis, as well as inflammation and tissue repair [1].

The recent characterization of molecules controlling the growth and function of blood vessels has clarified the complex system of vascular biology, and the discovery of specific markers for differentiating vascular and lymphatic endothelial cells has enabled further investigation of the functions of these two cell types. Lymphatic vessel formation depends largely on the proliferation, migration, and tube formation of lymphatic endothelial cells (LECs) [2,3]. As LECs are difficult to purify and culture, the molecular mechanisms of LEC functions are poorly understood. However, very recently, several specific markers and growth factors for LECs have been reported. In particular, LYVE-1 [4], Prox-1 [5], and podoplanin [6] have been identified as specific markers for LECs.

Vascular endothelial growth factor (VEGF) is a member of the platelet-derived growth factor superfamily and regulates endothelial functions, including vasodilation, proliferation, permeability, migration, and survival [7–10]. VEGF is also an important regulator of blood vessel growth and development [11–13]. VEGF exerts its effects through interactions with VEGF receptor (VEGFR) 1 (Flt-1) [14,15] and VEGFR2 (Flk-1/KDR) [16,17]. As tyrosine kinase receptors, VEGFR1 and VEGFR2 phosphorylate specific tyrosine residues in the SH2 domain of signaling molecules [18,19]. VEGFR1 undergoes weak ligand-dependent tyrosine phosphorylation, whereas VEGFR2 responds strongly. The differences in the properties of these receptors correspond to the diverse functions of VEGF [15,18]. VEGFR1 mediates cell migration and differentiation, whereas VEGFR2 mediates cell proliferation and survival [15,20–22]. LECs express two VEGFRs, VEGFR2 and VEGFR3, and thus LEC function is regulated by VEGF [1,3].

The major signaling pathway of VEGFR is thought to be the MAPK pathway [22,23], although signal transducer and activator of transcription (STAT) family members also function during VEGFR signaling [24–26]. In response to ligand binding, STATs are activated, dimerize, and translocate to the nucleus, where they bind specific target gene promoters [27–29].

Among the STAT family proteins, STAT3 is important for cell migration [30]. STAT3 is phosphorylated in various cell types by interleukin-6 family proteins, epidermal growth factor, platelet-

Abbreviations: VEGF, vascular endothelial growth factor; LEC, lymphatic endothelial cell; STAT, signal transducer and activator of transcription; p-STAT3, phosphorylated STAT3; HDMEC, human dermal microvascular endothelial cell; Ax, adenovirus vector; STAT3F, dominant-negative form of STAT3; STAT1F, dominant-negative form of STAT1.

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derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor, and leptin [27,31]. We have previously shown that STAT3 is essential for VEGF-induced human dermal microvascular endothelial cell (HDMEC) migration and tube formation [32]. Therefore, we hypothesized that STAT3 also regulates VEGF-induced LEC migration and tube formation. To test this hypothesis, we blocked STAT3 function in LECs through transfection with a dominant-negative STAT3 (STAT3F), using an adenovirus vector. Here, we show the first evidence that STAT3 regulates LEC function.

2. Materials and methods

2.1. LEC culture

Human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) were purchased from Lonza Walkersville, Inc. (Walkersville, MD) and were maintained in EGM-2 MV medium (Lonza Walkersville, Inc.).

2.2. Western blot analysis

LECs were harvested on ice with lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40) containing proteinase inhibitors (Sigma Chemical Co., St. Louis, MO). Western blot analysis was performed as previously described [32], using anti-STAT1, anti-STAT3 (BD Transduction Laboratories, Lexington, KY), anti-phospho-STAT1 (New England Biolabs, Beverly, MA), and anti-phospho-STAT3 antibodies (Cell Signaling, Beverly, MA).

Cytoplasmic and nuclear proteins were prepared from LECs by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL), according to the manufacturer's protocol.

2.3. Migration assay

LEC migration was evaluated with a modified Boyden chamber assay, as described previously [33]. Nucleopore polyvinylpyrrolidine-free polycarbonate membranes (8 μm; Neuro Probe, Inc., Gaithersburg, MD) were coated with type I collagen (Nitta Gelatin, Tokyo, Japan) for 30 min at room temperature and allowed to air dry. The membrane was placed over a 48-well chamber containing various concentrations of VEGF-A (R&D Systems, Minneapolis, MN) in culture medium. LECs (1×10^4 cells in 50 μl of medium) were seeded to the upper chamber of the apparatus. After 7 h, the upper surface of the membrane was scraped to remove non-migratory cells, and the filter was fixed and stained with hematoxylin and eosin. The total number of cells per well on the membrane was counted under a microscope.

2.4. Matrigel tube formation assay

A Matrigel tube formation assay was performed as previously described [32]. Matrigel (BD Biosciences Discovery Labware, Bedford, MA) prepared from the Engelbreth-Holm-Swarm tumor was added to 24-well plates and allowed to form a gel for 30 min at 37 °C. LECs were seeded at a density of 4×10^4 cells/well in 1 ml of medium with or without VEGF-A (33 ng/ml). After 24 h, the cells were observed microscopically. For quantification, the tube formations were traced, and tube length was calculated by Image-Pro Plus software.

2.5. Adenovirus vector (Ax)

The Ax-STAT1F and Ax-STAT3F vectors were prepared as described previously [32] and were used at a multiplicity of infection of 10 for the transfection of LECs. Ax carrying GFP (Ax-GFP) was used as a control vector.

2.6. Statistical analysis

The results are representative of three independent experiments. The p values were calculated with a two-sided Student's t-test.

3. Results

3.1. VEGF-A induces LEC migration and tube formation

VEGF-A-induced LEC migration was analyzed using a Boyden chamber assay (Fig. 1A). At a concentration of 33 ng/ml, VEGF-A enhanced LEC migration 2-fold compared with the control.

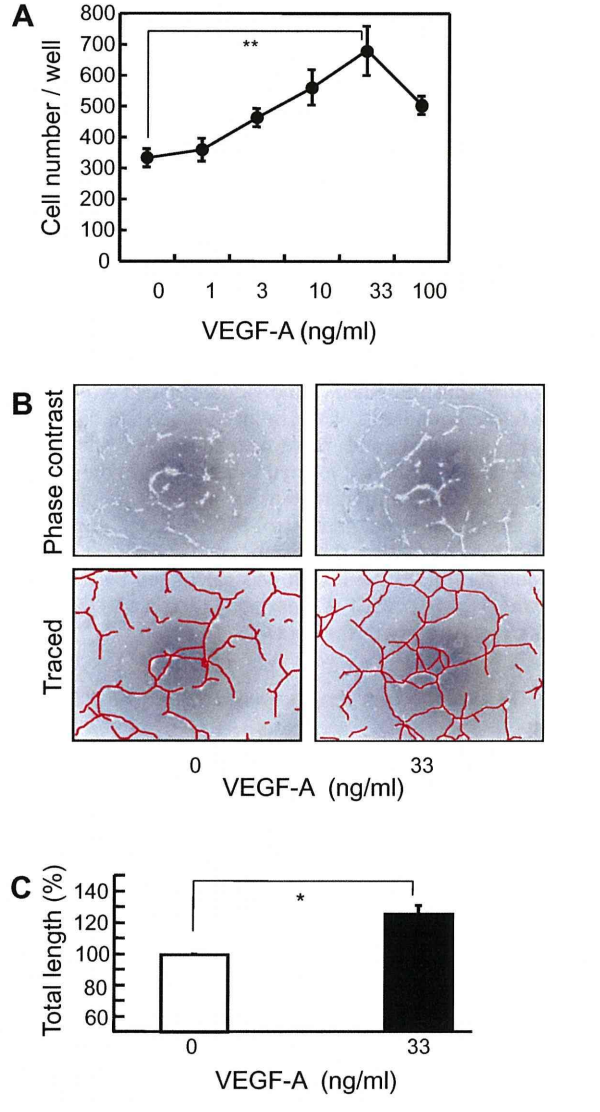

Fig. 1. VEGF-A-induced LEC migration and tube formation. (A) LEC migration was evaluated by a Boyden chamber assay. LECs were seeded on a membrane in the upper chamber of the apparatus, and VEGF-A was added to the lower chamber. After 7 h, the migrated cells were counted under a microscope. (B) Tube formation by VEGF-A-treated (33 ng/ml) and untreated LECs was assayed on Matrigel. After 24 h, the results were observed by phase contrast microscopy (upper panel), and the images were traced (lower panel). (C) The total tube length was calculated from the traced images using Image-Pro Plus software. The results are representative of three independent experiments. The p values were calculated with a two-sided Student's t-test (*$p < 0.05$; **$p < 0.01$).

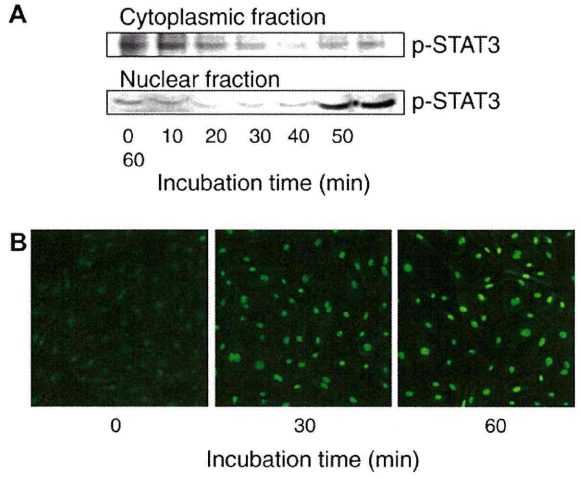

Fig. 2. VEGF-A-induced translocation of p-STAT3 to the nucleus. LECs were treated with VEGF-A (33 ng/ml), and the localization of p-STAT3 was analyzed. (A) Western blot analysis of p-STAT3 in the nuclear and cytoplasmic fractions of VEGF-A-treated LECs was performed at the indicated times. (B) VEGF-A-treated LECs were immunostained with anti-p-STAT3 antibody at the indicated times and observed by fluorescence microscopy.

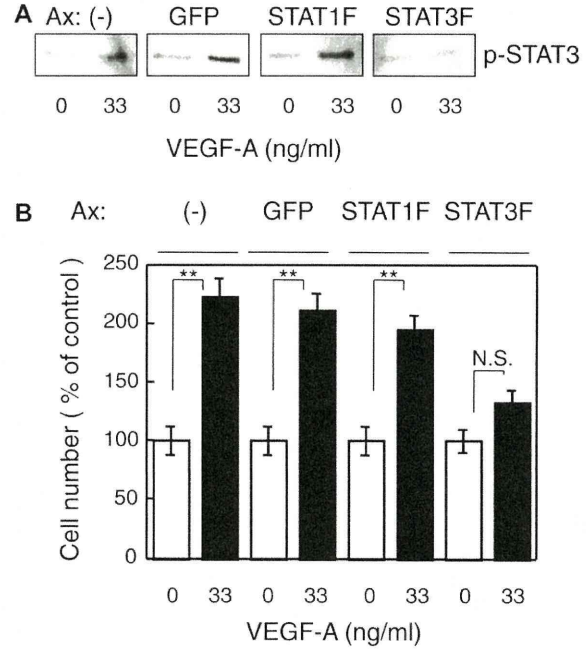

Fig. 3. Inhibition of VEGF-A-induced translocation of p-STAT3 and LEC migration by Ax-STAT3F. Ax-STAT1F and Ax-STAT3F were prepared as previously described [34]. Ax-GFP was used as a control. The transfection efficiency of Ax into LECs was approximately 100%, as determined by transfection with Ax-GFP. (A) At 24 h after transfection, the LECs were stimulated with VEGF-A (33 ng/ml) for 1 h, and the LEC nuclear fraction was prepared and subjected to Western blot analysis with anti-p-STAT3. (B) At 24 h after transfection, the LECs were allowed to migrate for 7 h with or without the addition of VEGF-A (33 ng/ml), as described in Fig. 1A. The results are representative of three independent experiments. The p values were calculated by a two-sided Student's t-test (NS, not significant; $^{**}p < 0.01$).

The ability of VEGF-A to induce LEC tube formation was assessed using Matrigel. Between 1 and 2 h after plating, the LECs began to rearrange or align themselves in the presence or absence of VEGF-A. By 12 h after the addition of VEGF-A, the LECs had differentiated into an expansive tube network, whereas the majority of the control cells remained as individual clusters or ovoid colonies. By 16 h, the boundaries of the induced tubes had become sharply defined, elongated, and more extended, and the majority of VEGF-A-treated LECs had formed tubes. At 24 h, the VEGF-A-treated LECs showed a more extensive network of interconnecting tubes, compared with the control LECs (Fig. 1B, upper panel). To quantify tube formation, the images were traced (Fig. 1B, lower panel), and the total tube length was calculated by Image-Pro Plus software (Fig. 1C). VEGF-A treatment increased LEC tube length by 25%, compared with the control.

3.2. VEGF-A translocates phosphorylated STAT3 (p-STAT3) to the nucleus in LECs

We next studied the mechanism of VEGF-A-induced LEC migration and tube formation. We have previously shown that STAT3 is involved in VEGF-A-induced HDMEC migration and tube formation [32]. Therefore, we examined whether STAT3 is also involved in LEC function. Although LECs expressed STAT3, the whole-cell p-STAT3 level did not change during VEGF-A treatment (data not shown). Given that phosphorylated STATs translocate to the nucleus, we determined whether VEGF-A treatment caused the translocation of p-STAT3 to the nucleus. With VEGF-A treatment of LECs, p-STAT3 was translocated from the cytoplasmic to the nuclear fraction (Fig. 2A). Immunostaining confirmed the translocation of p-STAT3 to the nucleus (Fig. 2B). In contrast, VEGF-A treatment did not affect the localization of phosphorylated STAT1 (data not shown).

3.3. STAT3F inhibits VEGF-induced nuclear translocation of p-STAT3

To further investigate the involvement of STAT3 in LEC function, we constructed adenovirus vectors (Ax) expressing dominant-negative mutants of STAT1 (Ax-STAT1F) and STAT3 (Ax-STAT3F), as described previously [32]. Ax-GFP was used as a negative control. The transfection efficiency of Ax into LECs was approximately 100%, determined by the transfection of Ax-GFP.

At 24 h after LECs were transfected with Ax, the translocation of p-STAT3 to the nucleus was analyzed on Western blots (Fig. 3A). The transfection of LECs with Ax-STAT3F almost completely inhibited the VEGF-A-induced translocation of p-STAT3 to the nucleus, whereas transfection with Ax-STAT1F or Ax-GFP had no effect on translocation.

3.4. STAT3F inhibits VEGF-A-induced LEC migration and tube formation

We used Ax-STAT3F to analyze whether STAT3 is essential for VEGF-A-induced LEC migration and tube formation. Transfection with Ax-STAT3F inhibited VEGF-A-induced LEC migration, whereas Ax-GFP or Ax-STAT1F had no effect (Fig. 3B). Transfection with Ax-STAT3F also inhibited VEGF-A-induced tube formation (Fig. 4A) and completely prevented the VEGF-induced increase in tube length compared with the control (Fig. 4B). These results indicate that STAT3 is essential for VEGF-A-induced LEC migration and tube formation.

4. Discussion

In this study, we showed that VEGF-A induces LEC as well as HDMEC migration and enhances tube formation via STAT3 signaling. VEGF-induced angiogenesis has previously been reported; however, the finding that STAT3 plays a central role in lymphangiogenesis induced by VEGF may help to elucidate the pathophysiology of cancer progression and metastasis, inflammatory diseases, and other lymphatic diseases. Blood vessel regeneration is a vital process in

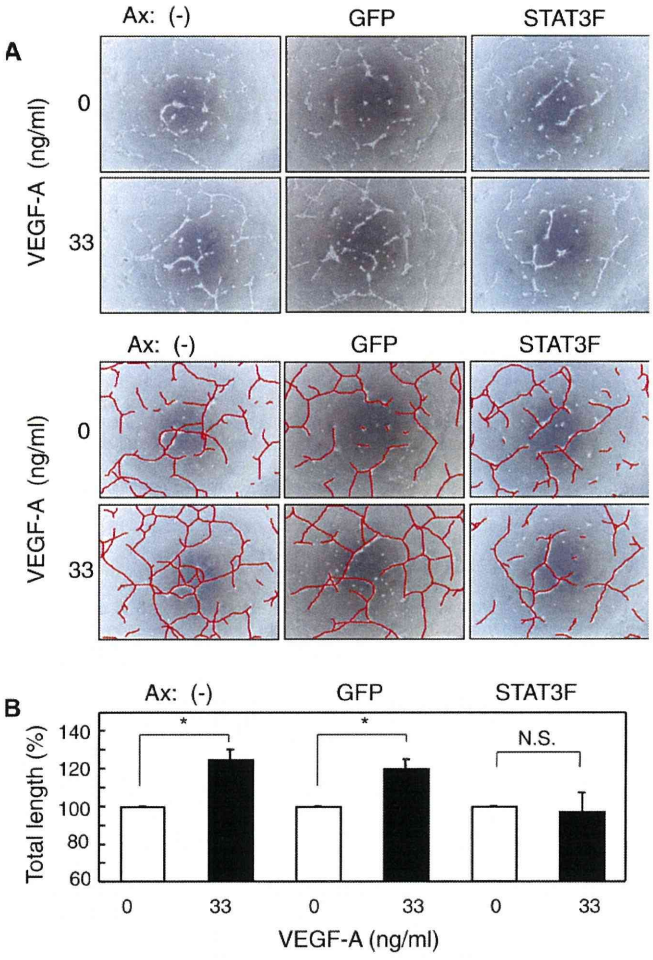

Fig. 4. Inhibition of VEGF-A-induced LEC tube formation by Ax-STAT3F. At 24 h after transfection, the LECs were allowed to form tubes on Matrigel for another 24 h in the presence or absence of VEGF-A (33 ng/ml), as described in Fig. 1B. (A) Tube formation by LECs on Matrigel was observed by phase contrast microscopy (upper panel), and the images were traced (lower panel). (B) The total tube length was calculated from the traced images using Image-Pro Plus software. The results are representative of three independent experiments. The p values were calculated by a two-sided Student's t-test (NS, not significant; $^*p < 0.05$).

skin wound healing and is regulated by various cytokines and growth factors. Among these, the most important is VEGF, which has been shown to directly induce blood vessel formation *in vivo* [34,35] and to induce vascular endothelial cell migration and blood vessel regeneration *in vitro* [36,37]. Compared with basic fibroblast growth factor, VEGF strongly induces the migration of human umbilical vein endothelial cells, even at low concentrations [38]. Thus, a major function of VEGF is the induction of endothelial cell migration, including LEC migration, as LECs express VEGFR2 and VEGFR3 [1]. VEGF-A binds VEGFR2 on LECs to activate LEC functions [39]. Very recently, VEGF-C and VEGF-D were shown to bind and activate VEGFR3 on LECs [40,41].

Tyrosine phosphorylation of STAT is necessary, but not sufficient, for its transcriptional activity. It is believed that chaperone proteins are required to assist the nuclear translocation of STATs, because STATs lack a nuclear localization signal [42]. In this study, we showed that the translocation of p-STAT3 into the nucleus is essential for triggering LEC migration and tube formation. However, the whole-cell p-STAT3 level was not altered by VEGF-A. Similarly, p-STAT3 translocation is essential for VEGF-induced HDMEC migration and tube formation, and the total p-STAT3 level is not altered in HDMECs [32]. Thus, the localization of p-STAT3, and not the total p-STAT3 level, is important for migration and tube formation by LECs and HDMECs. In aortic endothelial cells, VEGF induces the phosphorylation of STAT1, STAT3, and STAT6 [24], and phosphorylated STAT1 and STAT6, but not p-STAT3, are translocated into the nucleus. This illustrates that the role of STAT3 in VEGF signaling varies among endothelial cell types.

Two pathways have been reported for the phosphorylation of STATs: Janus kinase and the intrinsic tyrosine kinase of growth factor receptors [24]. However, we previously reported that JAK1, JAK2, and Tyk2 were not phosphorylated by VEGF-A in HDMECs [32], and in the present study, JAK1, JAK2, and Tyk2 were not phosphorylated by VEGF-A in LECs (data not shown). This suggests that the intrinsic tyrosine kinase activity of VEGFR phosphorylates STAT3 in LECs. In bovine aortic endothelial cells, VEGFR2 intrinsic tyrosine kinase activity was reported to be involved in STAT1 phosphorylation [24]. Thus, the activation mechanisms of STAT3 differ among endothelial cell types.

VEGFR1 and VEGFR2 provide essential regulatory signals for blood vessel formation in mammals. In previous studies, VEGFR1- or VEGFR2-deficient mouse embryos died at E8.5–9.0 [21] or E8.5 [13], respectively, from a severe deficiency in vascular formation associated with strong hematopoietic impairment. STAT3-deficient mouse embryos also died at approximately E7.0 [43]. However, LECs are not identified until day E9.5, it still remains unclear whether STAT3 play some role in LEC embryogenesis. Transgenic mice with LEC specific Cre recombinase could be used to investigate its function. In the present study, STAT3 was shown to be essential for VEGF-induced LEC migration and tube formation. Although angiogenesis and vascular formation have not been fully investigated, these findings suggest that lymphangiogenesis is impaired during development in STAT3-deficient mouse embryos, and this may account for the early mortality.

Acknowledgments

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Interactions between myofibroblast differentiation and epidermogenesis in constructing human living skin equivalents

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ABSTRACT

Background: During skin wounding and healing, skin homeostasis is interrupted. How the altered epithelial–mesenchymal interactions influence scar formation and epidermogenesis should be investigated using three-dimensional models that are similar to *in vivo* structures.
Objective: In this study, we assessed the effects of epithelial–mesenchymal interactions on myofibroblast differentiation and how myofibroblasts influence epidermogenesis using a human living skin equivalent (LSE) model.
Methods: We constructed a fibroblast-populated type I collagen gel upon which LSEs were formed by seeding with normal human keratinocytes. Samples of the collagen gel and LSEs were collected at different time points. Myofibroblast differentiation, epidermal differentiation, and proliferation status were investigated immunohistochemically. Several measures were taken to suppress α-smooth muscle actin (α-SMA) expression to determine the effects of myofibroblasts on epidermogenesis, including the addition of basic fibroblast growth factor or a transformation growth factor-β (TGF-β) kinase inhibitor to the culture medium and the inclusion of an amniotic membrane (AM) in the dermal matrix.
Results: The myofibroblast/fibroblast ratio in the fibroblast-populated collagen gel kept rising during culture. In the LSEs, most fibroblasts were α-SMA-negative, except for those along the dermal–epidermal junction. The suppression of α-SMA expression enhanced epidermal differentiation and decreased TGF-β1 expression in the epidermis. The inhibition of TGF-β kinase completely suppressed α-SMA expression in the dermal matrix.
Conclusions: Epidermogenesis suppressed α-SMA expression in the fibroblast-rich dermal matrix, except near the dermal–epidermal junction. The α-SMA-positive cells at the dermal–epidermal junction contributed to the hyperproliferative phenotype of the epidermis. In contrast, the hyperproliferative epidermis expressed more TGF-β1, which is responsible for myofibroblast differentiation.

1. Introduction

During skin wound healing and tissue repair, fibroblasts from adjacent, intact dermis are activated and migrate to the fibrin clot, where they differentiate into α-smooth muscle actin (α-SMA)-expressing myofibroblasts in response to growth factors such as transforming growth factor-β1 (TGF-β1), mechanical tension, and the extracellular matrix (ECM) [1,2]. Myofibroblasts show enhanced ECM secretion, and they exert a retractile force upon the ECM, which contributes to scar formation [3,4]. After wounding, keratinocytes at the wound margin are activated and begin to migrate to the newly formed dermal matrix to reestablish the skin integument. Features of the dermal matrix during wound healing and in newly healed skin, hypertrophic scars, or keloids include keratin 6-, 16-, and 17-expressing keratinocytes, infiltration by various inflammatory cells, and myofibroblasts, which may remain in the dermal matrix for a long time [5–10]. However, the basement membrane is not well developed [11].

Myofibroblasts play important roles in skin wound healing and scar formation. Until now, most studies have been focused on dermal events. In recent years, epithelial–mesenchymal interactions in wound healing and scar formation have drawn much attention. Co-culture of fibroblasts and keratinocytes up-regulated TGF-β1 expression in fibroblasts, increased ECM deposition, and induced α-SMA expression in fibroblasts of close proximity [12]. In other studies, keratinocytes suppressed the proliferation of co-cultured

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fibroblasts and ECM production, and the medium from subconfluent or confluent keratinocytes exerted differing effects on co-cultured fibroblasts and ECM synthesis [13,14]. In these reports, keratinocytes, whether differentiated or undifferentiated, confluent or subconfluent, or separated from or in physical contact with fibroblasts, had different profiles of secreted cytokines, which may explain their seemingly paradoxical effects on co-cultured fibroblasts. Furthermore, cells grown as a monolayer or in a three-dimensional culture may adopt different phenotypes and functions. Thus, investigations of monolayer cell cultures may not be sufficient to explain *in vivo* events.

Wound healing is mediated by interactions between various cells. In the middle or late phase of wound healing, interactions between fibroblasts and keratinocytes dominate, especially in wound remodeling and scar formation. In this study, we established a fibroblast-populated type I collagen gel and living skin equivalents (LSEs) by seeding keratinocytes on the gel. Fibroblasts embedded in the collagen came into contact with the overlying stratified epidermis at the dermal–epidermal junction. Such three-dimensional models may better reveal what occurs during wound healing and scar formation. In the models, we investigated fibroblast–myofibroblast events and their interplay with the epidermis. Furthermore, we investigated the influence of myofibroblasts on epidermogenesis by suppressing myofibroblast differentiation.

These LSE models were histologically and molecularly a model of regenerating skin in wound healing [15,16]. We therefore developed a new skin model in which de-epithelialized human amnion was inserted between the dermal and epidermal compartments. This new skin model exhibited a well-developed basement membrane and a better differentiated and stratified epidermis [17]. Using this model, we investigated how the epidermal phenotype influences myofibroblast differentiation, and the importance of a well-developed basement membrane in skin regeneration and maturation and the prevention of scar formation.

2. Materials and methods

2.1. Cell culture

Normal human epidermal keratinocytes were isolated from healthy human skin and cultured under serum-free conditions, as described previously [18,19]. The cells were used for LSE cultures in their fourth passage. Fibroblasts were isolated from healthy human skin and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS). Fifth-passage cells were used to construct a fibroblast-populated collagen gel or LSE model. The keratinocytes and fibrobalsts are from two different individuals. And the cells are being used in our lab for years.

All procedures involving human subjects received prior approval from the ethics committee of Ehime University School of Medicine, Toon, Ehime, Japan. All subjects provided written informed consent.

2.2. Preparation of the fibroblast-populated type I collagen gel and LSE

The methods used to prepare the collagen gel and LSE were described previously [20]. Briefly, a collagen gel was prepared by mixing six volumes of ice-cold porcine collagen type I solution (Nitta Gelatin, Osaka, Japan) with one volume of 8× DMEM (Gibco), ten volumes of 1× DMEM, supplemented with 20% FCS, and one volume of 0.1 N NaOH. The final concentration of collagen was 0.8 mg/mL. A total of 1 mL of the mixture was added to each culture insert (Transwel-COL, membrane pore size 3 μm; Costar, Corning, NY, USA) in a six-well Costar culture plate (Corning). Following polymerization of the gel in the inserts at 37 °C, two volumes of fibroblast suspension solution (5×10^5 cells/mL in 1× DMEM supplemented with 10% FCS) were added to eight volumes of the collagen solution, after which 3.5 mL of the fibroblast-containing collagen solution was applied to each insert. When the fibroblast-containing gel polymerized, DMEM supplemented with 10% FCS and ascorbic acid (final concentration 50 ng/mL) was added. The culture medium was changed twice per week. The gel was collected at different time points, processed, and embedded in paraffin for histological and immunohistochemical (IHC) study or used to construct the LSEs.

Five days after the dermal component was prepared, 6.0×10^5 keratinocytes in 60 μL of MCDB 153 type II were seeded onto the concave surface of the contracted gel. The keratinocytes were kept submerged in culture medium for 2 days. When the keratinocytes reached confluence, the LSE was lifted to an air–liquid interface and cornification medium [20] was added. This medium was changed every other day. At different time points after airlift, the LSEs were harvested. Basic fibroblast growth factor (bFGF; provided by Kaken Pharmaceutical Co., Tokyo, Japan) at 33 ng/mL or SB431542 (inhibitor of TGF-β receptor kinase; Sigma–Aldrich, St. Louis, MO, USA) at 10 μM was added to the culture medium of the LSEs in the test groups. The LSE samples were fixed in 20% formalin and embedded in paraffin for histological and IHC study. Some LSE samples were also processed for electron microscopy.

The amniotic membrane (AM)LSE was built by overlaying a de-epithelialized AM on the contracted collagen gel and seeding keratinocytes on the epithelial side of the AM. The timing of the airlift and culture medium were the same as for the AM-free LSE. Procurement and processing of the AM were as described previously [17]. Two weeks after airlift, the AMLSEs were harvested and processed for histological and IHC analyses.

We performed at least three independent studies, which produced similar results. A representative experiment is shown in the figures.

2.3. Histological and IHC staining

Paraffin-embedded LSE samples were sectioned at 6 μm and stained with hematoxylin and eosin. For IHC staining, a Histofine Simple Stain MAX-PO (M) kit (Nichirei, Tokyo, Japan) was used according to the manufacturer's instructions. The antibodies used in this study were V9 for vimentin (Dako), 1A4 for α-SMA (Dako), LL025 for keratin 16 (NeoMarkers), LHP1 for keratin 10 (NeoMarkers), and 1D11 for TGF-β1 (R&D Systems). Images were obtained using an Olympus AX80 microscope coupled with an Olympus DP50 digital camera (Olympus, Tokyo, Japan). We performed at least three independent studies, which gave similar results. A representative experiment is shown in the figures.

2.4. Transmission electron microscopy (TEM)

Specimens were fixed in 0.1% tannic acid containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, washed with phosphate buffer, post-fixed with 1% osmium tetroxide in phosphate buffer for 2 h, washed with 0.25 M sucrose, dehydrated through a graded ethanol series, and embedded in Epon resin.

Ultrathin sections (<60–80 nm) were prepared using an Ultracut S (Leica, Solms, Germany), double-stained with uranyl acetate and lead citrate, and examined with a JEM 1230 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV. We performed at least three independent studies, which produced similar results. A representative experiment is shown in the figures.