

Original article

Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes

Background: House dust mites produce serine and cysteine proteases. Mite-derived proteases have been suggested to be involved in the pathogenesis of allergies; however, whether mite-derived serine protease activity can stimulate keratinocytes remains unknown.

Methods: We examined the activation of primary human keratinocytes by serine protease-rich extract of whole mite culture and compared with that by recombinant group 1 allergens (rDer f 1 and rDer p 1), which exclusively exhibit cysteine protease activity.

Results: Protease activity of whole mite culture extract (WCE), rDer f 1 and rDer p 1 induced the release of IL-8 and granulocyte-macrophage colony-stimulating factor. Protease activity of WCEs induced a significant upregulation of their mRNA expression but rDer f 1 had much less effect. Protease activity of the WCE stimulated intracellular Ca^{2+} mobilization but rDer f 1 and rDer p 1 did not. The mobilization induced by agonists for the human protease-activated receptor (PAR)-2, an agonist peptide or trypsin, was diminished by pre-incubation of keratinocytes with WCE. rDer f 1 inefficiently cleaved a synthetic N-terminal peptide of PAR-2 at different sites from trypsin, but the resultant peptides did not stimulate the release of interleukin-8.

Conclusions: The results suggest that mite-derived serine protease activity may contribute to the pathogenesis of atopic dermatitis by activating keratinocytes via PAR-2 activation but cysteine protease activity of Der f 1 and Der p 1 acts via another mechanism.

T. Kato¹, T. Takai¹, T. Fujimura²,
H. Matsuoka³, T. Ogawa^{1,4},
K. Murayama², A. Ishii³, S. Ikeda^{1,4},
K. Okumura^{1,5}, H. Ogawa^{1,4}

¹Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo, Japan;

²Division of Proteomics and Biomolecular Science, Biomedical Research Center, Juntendo University School of Medicine, Tokyo; ³Division of Medical Zoology, Jichi Medical University, Tochigi;

⁴Department of Dermatology, Juntendo University School of Medicine, Tokyo; ⁵Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Key words: atopic dermatitis; cysteine protease; house dust mite; keratinocyte; proinflammatory cytokine response; protease-activated receptor-2; serine protease.

Toshiro Takai
Atopy (Allergy) Research Center
Juntendo University School of Medicine
2-1-1 Hongo, Bunkyo-ku
Tokyo 113-8421
Japan

Accepted for publication 3 February 2009

House dust mites belonging to two species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major sources of allergens associated with allergic diseases such as asthma, rhinitis and atopic dermatitis. More than 20 groups of house dust mite-derived proteins have been identified as allergens, which have IgE reactivity in allergic patients (1). Among them, group 1 and group 2 allergens are major allergens based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract. At least four of the 20 groups are proteases. Group

1 allergens belong to the papain-like cysteine protease family, and group 3, 6 and 9 allergens are serine proteases.

Molecules produced by allergen-producing organisms have been suggested to be involved in the pathogenesis of allergic diseases through sensitization and/or exacerbation via IgE-independent mechanisms (2–6). Cysteine protease activity of the group 1 allergens, Der f 1 and Der p 1, reduces the barrier functions of tissue barriers, cleaves and/or interacts with various molecules including cell-surface molecules and endogenous protease inhibitors, modulates the functions of various cells, and induces Th2-skewed sensitization (2, 3, 7–11). Mite-derived serine proteases also have biological activities including the ability to disrupt tight junctions, stimulate airway and ocular conjunctival epithelial cells, cleave an endogenous protease inhibitor, and so on (12–15).

The skin epidermis and airway epithelium are located at the interface between the body and environment and have critical roles in the response to various stimuli

Abbreviations: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AP, agonist peptide; GM-CSF, granulocyte-macrophage colony-stimulating factor; PAR, protease-activated receptor; rDer f 1, recombinant Der f 1-N53Q; rDer f 1-C35S, a mutant rDer f 1 with no protease activity; rDer p 1, recombinant Der p 1-N52Q; RP, reversed peptide; WCE, whole mite culture extract of *Dermatophagoides farinae*.

during the sensitization and effector phases in allergic diseases (16, 17). House dust mite allergens are important factors in atopic dermatitis, as well as asthma, rhinitis, and allergic conjunctivitis, and are present on the surface of human skin (18, 19). Recently, we have reported the release of interleukin (IL)-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) from keratinocytes stimulated with recombinant mite group 1 allergens, which show cysteine protease activity alone, and its inhibition by cystatin A, a skin-derived protease inhibitor (20, 21); however, whether mite-derived serine protease activity can stimulate keratinocytes remains unknown. Only one report (22) has investigated whether *D. pteronyssinus* mite extract stimulates keratinocytes and it described that this extract did not stimulate the expression of IL-8 and GM-CSF in keratinocytes. Here, we examined the activation of keratinocytes stimulated with a whole mite culture extract (WCE) of *D. farinae* with significant serine protease activity and compared with that by recombinant mite group 1 allergens, which exclusively exhibit cysteine protease activity.

Materials and methods

WCE

The WCE, which can induce atopic dermatitis in NC/Nga mice, was prepared from a whole culture of *D. farinae*, as previously described (23), as follows. Mites were cultured for 2 months. Phosphate-buffered saline (PBS) was added to the whole culture and gently rotated for 30 min. After centrifugation, the supernatant was filtered for sterilization, and stored at -80°C prior to use. The protein concentration was determined by the Bradford procedure with a protein assay kit with bovine IgG as the standard (Bio-rad, Richmond, CA, USA).

Recombinant mite group 1 allergens

Mature forms of Der f 1-N53Q (rDer f 1), Der f 1-N53Q (rDer p 1) (24), and rDer f 1-C35S, a mutant rDer f 1 without protease activity (25), were used. They have no yeast-derived hyperglycosylation and have molecular weights identical to natural allergens. A precursor form of rDer f 1-C35S was prepared by site-directed mutagenesis and converted to its mature form. The mature rDer f 1-C35S was purified with column chromatography and yeast-derived protease activity was removed.

Protease activity

Protease activity was measured as previously described (26). Time courses of the digestion of two fluorogenic substrates, butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA) and butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA) (Peptide Institute, Osaka, Japan), were monitored.

Synthetic peptides

An agonist peptide (AP) for the human protease-activated receptor (PAR)-2 (PAR-2 AP; SLIGKV-NH₂) and a peptide

with the reversed sequence of the PAR-2 AP [PAR-2 reversed peptide (RP); VKGILS-NH₂] were purchased from Bachem (Bubendorf, Switzerland). The other peptides were synthesized by a peptide synthesizer and purified with reverse-phase high-performance liquid chromatography (HPLC).

Culture and stimulation of keratinocytes

Primary human keratinocytes from infant foreskins (Cascade Biologics, Portland, OR, USA) at the third or second passage and a human keratinocyte cell line, HaCaT, were cultured and stimulated as previously described (20), briefly as follows. Keratinocytes grown to 80% confluency were cultured in basal medium without supplements or fetal calf serum (FCS), HuMedia (Kurabo, Osaka, Japan) for primary human keratinocytes or Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) for HaCaT, for 12–24 h before stimulation, and then washed and incubated with the stimulants. The WCE, rDer p 1, and rDer f 1 were pre-incubated with 5 mM L-cysteine at neutral pH to regenerate cysteine protease activity and diluted in the basal medium. In some experiments, the stimulants were pretreated with 0.125 mM E-64 (Peptide Institute, Osaka, Japan) and/or 2.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma) and diluted. Final concentrations of E-64 and AEBSF were 1.25 and 25 μM , respectively.

ELISA and real-time PCR

Concentrations of IL-8 and GM-CSF in the culture supernatants of keratinocytes were measured with ELISA kits (DuoSet; R&D Systems, Minneapolis, MN, USA). Total RNA was extracted from keratinocytes using STAT-60 reagent (Tel-Test, Friendswood, TX, USA). First-strand cDNA was synthesized and RNA complementary to the cDNA was removed by treatment with RNase H. Real-time quantitative PCR was performed with the TaqMan method using an ABI 7500 (Applied Biosystems, Piscataway, NJ, USA).

Intracellular Ca²⁺ mobilization

Keratinocytes were detached from culture plates by Cell Dissociation Solution (CDS; Sigma), a nonproteolytic cell dissociation solution, after being washed with PBS. To facilitate detachment, bovine α -chymotrypsin (1 μM ; Sigma) (27) was added to CDS-treated keratinocytes for 5 min at room temperature. Detached cells were washed twice with an assay buffer (PBS containing 0.2% bovine serum albumin (BSA), 0.1% glucose and 30 μM CaCl₂), resuspended to a concentration of 2×10^6 cells/ml, and diluted with an equal volume of 4 mM Fura-2 AM. After incubation with Fura-2 AM for 1 h at 37°C , free Fura-2 AM was removed by washing cells three times and the cells were resuspended in the assay buffer at a concentration of $0.5\text{--}1 \times 10^6$ cells/ml for stimulation. The suspension of the loaded cells was transferred to quartz cuvettes with a small magnetic stirrer and placed in a Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) at 37°C . Fura-2 fluorescence was measured at 340 nm and 380 nm (excitation) and 510 nm (emission). The WCE was passed through a NAP-5 column (GE Healthcare, Buckinghamshire, UK) for buffer exchange to PBS before use. The buffer-exchanged WCE, rDer p 1, and rDer f 1 were pre-incubated with 5 mM L-cysteine. In some experiments, the stimulants were pretreated with E-64 and/or AEBSF. After establishing a stable baseline, each of the enzymes or peptide was added to the loaded cells in the cuvettes.

Peptide cleavage analysis

A peptide corresponding to residues 32–45 in the human PAR-2 sequence (200 μ M) was synthesized by a peptide synthesizer and purified. The peptide was incubated with rDer f 1 (50 nM) and trypsin (5 nM) as a control, which is known to cleave PAR-2 at the activating site, in PBS at 37°C for 30, 60, 120 and 240 min. A 5 mM rDer f 1 was pre-activated with 5 mM L-cysteine and added to the reaction (1/100 dilution). The peak fractions were subjected to N-terminal sequencing and mass spectrometry to determine the sequences of peptides generated.

Results

Protease activity of WCE, rDer f 1, and rDer p 1

We analyzed the protease activity of WCE and recombinant mite group 1 allergens, rDer f 1 and rDer p 1, by conducting assays using synthetic fluorogenic substrates and protease inhibitors (Fig. 1). Boc-QAR-MCA and Boc-QGR-MCA are typical substrates suitable for measuring mite-derived cysteine and serine protease activities, respectively (26). The inhibitors, E-64 and AEBSF, are irreversible class-specific inhibitors for cysteine and serine proteases, respectively.

Treatment of WCE with L-cysteine resulted in a small increase in the cleavage of Boc-QAR-MCA, E-64 inhibited the increase (approximately 20% inhibition), and AEBSF exhibited approximately 80% inhibition of the cleavage overall (Fig. 1A, upper panel). Cleavage of Boc-QGR-MCA by WCE was not affected by treatment with L-cysteine, and AEBSF, but not E-64, inhibited it almost completely (Fig. 1B, upper panel). Without treatment with L-cysteine, rDer f 1 and rDer p 1 showed almost no activity against the two substrates (Fig. 1, middle and lower panels). When their catalytic thiol

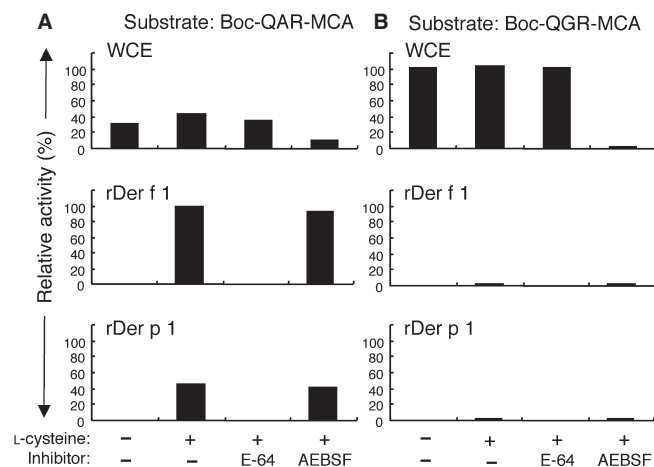


Figure 1. Protease activity of whole mite culture extract (WCE), rDer f 1, and rDer p 1. Substrates were Boc-QAR-MCA (A) and Boc-QGR-MCA (B). Fluorescence intensity relative to that of rDer f 1 activated with L-cysteine for Boc-QAR-MCA is shown. Data are representative of two independent experiments.

residue was regenerated by treatment with L-cysteine, they showed efficient cleavage of Boc-QAR-MCA (Fig. 1A, middle and lower panels) but relatively inefficient cleavage of Boc-QGR-MCA (Fig. 1B, middle and lower panels), and E-64, but not AEBSF, inhibited the cleavage completely (Fig. 1, middle and lower four panels). Thus, WCE exhibited mainly serine protease activity and minor cysteine protease activity, while rDer f 1 and rDer p 1 exhibited cysteine protease activity alone.

WCE, rDer f 1, and rDer p 1 induced release of IL-8 and GM-CSF

WCE (10 μ g/ml), rDer f 1 (100 nM) and trypsin (5 and 50 nM) promoted the release of IL-8 (Figs 2 and 3A,

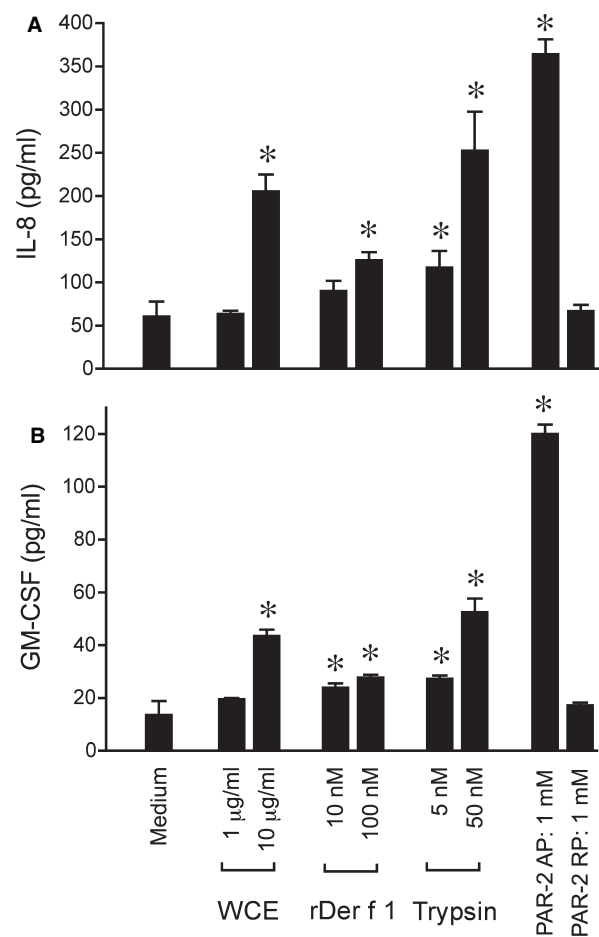


Figure 2. Release of interleukin (IL)-8 (A) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (B) from primary human keratinocytes stimulated with WCE and rDer f 1. Culture supernatants were recovered at 48 h. PAR-2 AP: agonist peptide of PAR-2. PAR-2 RP: peptide with the reversed sequence of PAR-2 AP. The data represent the mean $\pm$ SD for three wells of culture plates. The significance of the differences was determined with one-way ANOVA and Tukey's *post hoc* test. A value of $*P < 0.05$ compared with the control (Medium) was regarded as significant. Data are representative of more than three independent experiments.

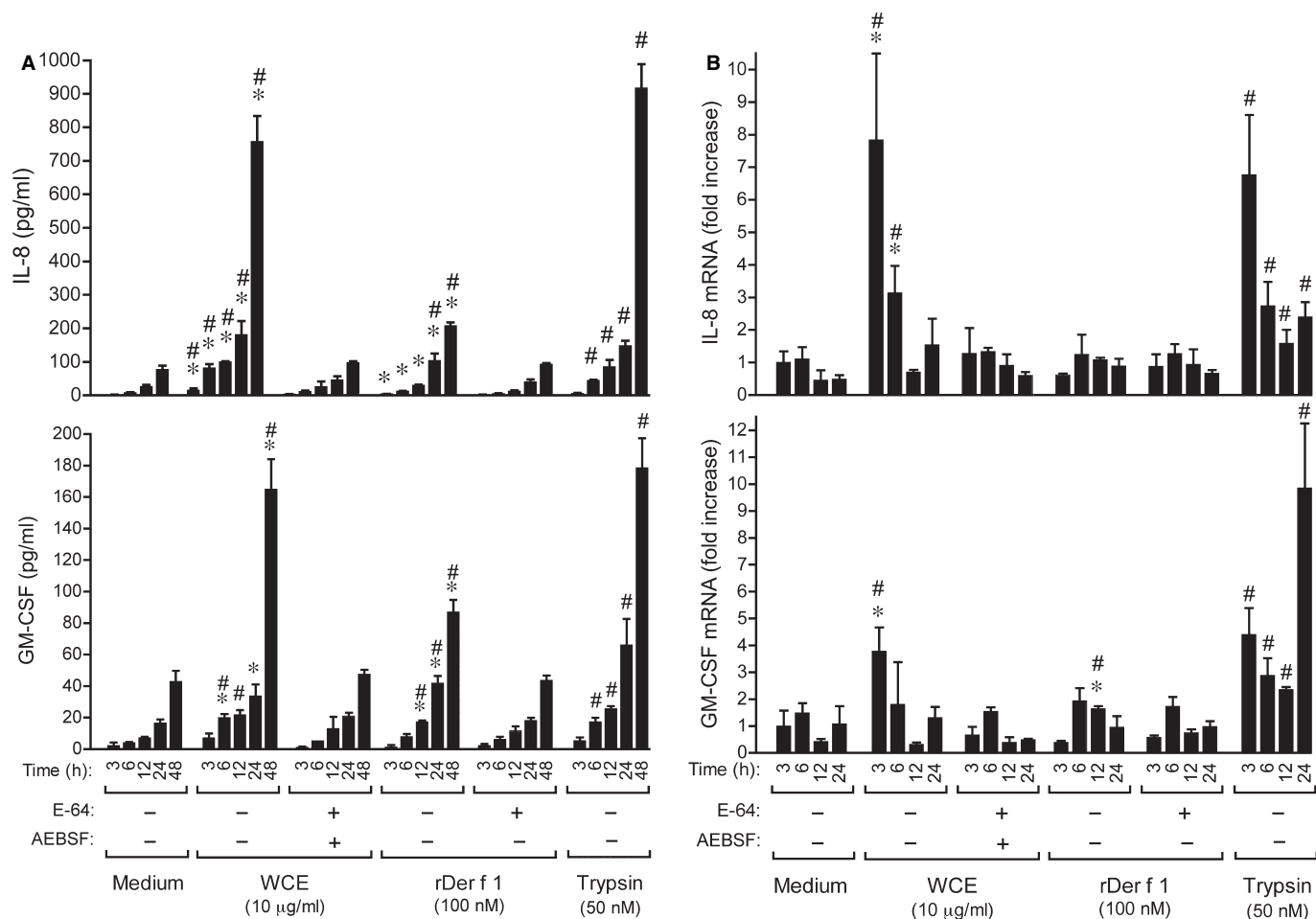


Figure 3. Time course of release (A) and mRNA levels (B) of IL-8 and GM-CSF in primary human keratinocytes stimulated with WCE and rDer f 1. In (B), the mRNA level was normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase and changes in gene expression were indicated as fold increases relative to the mean of untreated control wells (*Medium*) 3 h after the beginning of stimulation. The data represent the mean $\pm$ SD for three wells of culture plates. The significance of differences was determined with one-way ANOVA and Tukey's *post hoc* test. A value of $P < 0.05$ was regarded as significant. Data are representative of more than three independent experiments. * $P < 0.05$ compared with the control (*Medium*) and the sample with inhibitors among the three groups at each incubation time. # $P < 0.05$ compared with the control (*Medium*) among the six groups.

upper panels) and GM-CSF (Figs 2 and 3A, lower panels) from primary human keratinocytes. The final concentrations of L-cysteine were less than 50 μ M. L-cysteine at these concentrations did not affect the release of IL-8 and GM-CSF (data not shown).

WCE more significantly upregulated the mRNA expressions of IL-8 and GM-CSF than rDer f 1

The expressions of IL-8 and GM-CSF at the transcriptional level were evaluated by real-time quantitative PCR (Fig. 3B). The WCE and trypsin significantly upregulated the mRNA expression of IL-8 (Fig. 3B, upper panel) and GM-CSF (Fig. 3B, lower panel), with a peak at 3 h. In contrast, rDer f 1 had much less effect on mRNA expression than WCE and trypsin (Fig. 3B).

Protease activity of WCE, rDer f 1, and rDer p 1 induced release of IL-8 and GM-CSF

Primary human normal keratinocytes were stimulated in original basal medium, HuMedia, containing 0.15 mM CaCl_2 (Figs 2, 3 and 4A) or calcium-added basal medium containing 1.35 mM CaCl_2 (Fig. 4B).

Upregulation of the release of IL-8 and GM-CSF was inhibited by AEBSF for WCE and trypsin and by E-64 for rDer f 1 and rDer p 1 (Figs 3A and 4A,B), and rDer f 1-C35S, a mutant rDer f 1 without protease activity, exhibited no effect (Fig. 4A,B, left panels). PAR-2 AP, but not PAR-2 RP, upregulated the release in the absence or presence of E-64 or AEBSF (Figs 2 and 4A,B, right panels). In primary keratinocytes, IL-8 release in response to mite proteases was

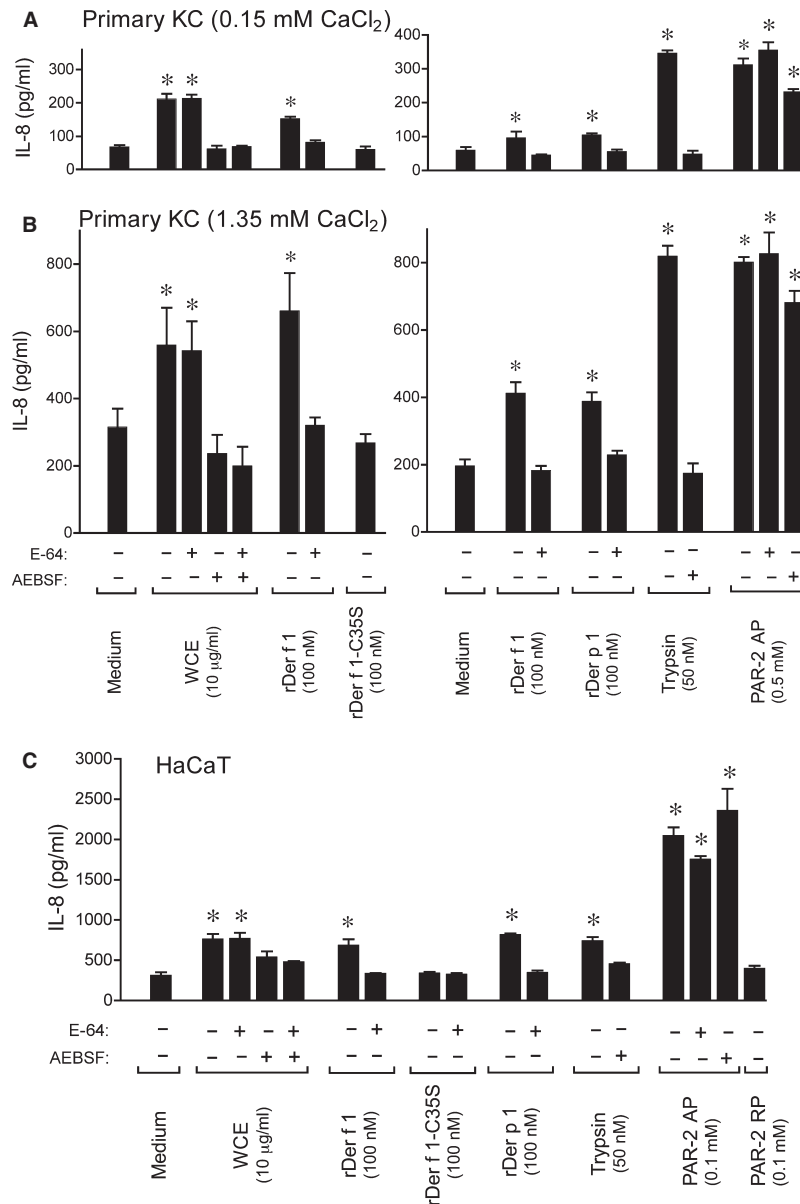


Figure 4. Effect of class-specific protease inhibitors on the release of IL-8 from primary human keratinocytes (A and B) and a human keratinocyte cell line, HaCaT, (C) stimulated with WCE, rDer f 1, and rDer p 1. Culture supernatants were recovered at 48 h. rDer f 1-C35S: a mutant rDer f 1 without protease activity. PAR-2 AP: agonist peptide of PAR-2. PAR-2 RP: peptide with the reversed sequence of PAR-2 AP. The data represent the mean $\pm$ SD for three wells of culture plates. The significance of differences was determined with one-way ANOVA and Tukey's *post hoc* test. A value of $*P < 0.05$ compared with the control (Medium) was regarded as significant. Data are representative of more than three independent experiments. Primary KC: primary human keratinocytes.

higher in keratinocytes stimulated in medium containing higher Ca^{2+} concentration (Fig. 4B), which induces differentiation, than in medium containing lower Ca^{2+} concentration (Fig. 4A). Protease inhibitors inhibited the upregulation of the mRNA expression of IL-8 (Fig. 3B, upper panel) and GM-CSF (Fig. 3B, lower panel) by WCE and trypsin. IL-8 release was also dependent on the serine protease activity of WCE and the cysteine protease activity of rDer f 1 and rDer

p 1 in the human keratinocyte cell line, HaCaT (Fig. 4C).

Protease activity of WCE, but not rDer f 1 and rDer p 1, induced the mobilization of Ca^{2+}

In the Ca^{2+} mobilization assay (Fig. 5), keratinocytes responded to PAR-2 AP (Fig. 5A), trypsin (Fig. 5C), and WCE (Fig. 5F), but not to PAR-2 RP (Fig. 5B), rDer f 1

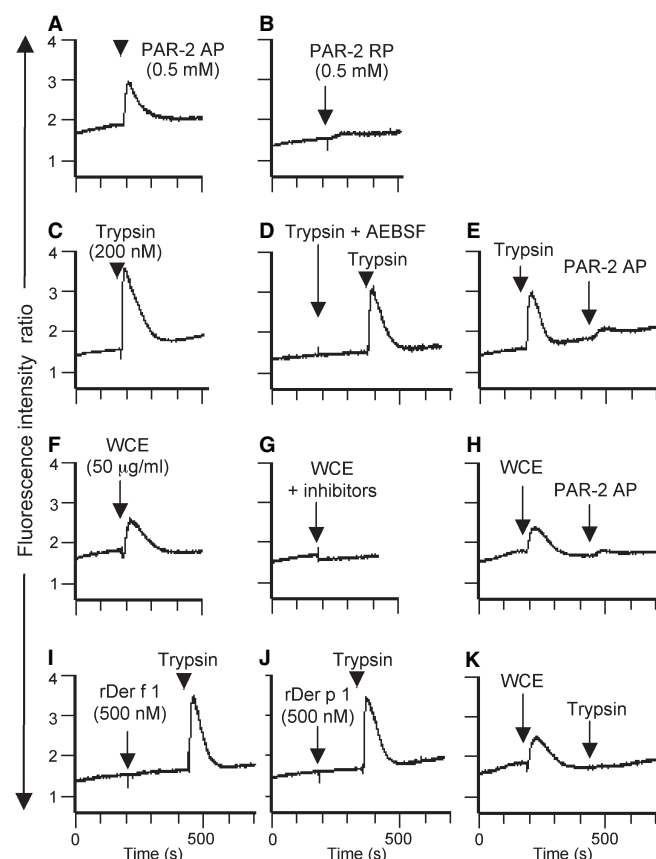


Figure 5. Intracellular Ca^{2+} mobilization. Detached primary human keratinocytes were used for the assays. The response was presented as relative fluorescence intensity at 510 nm (emission) obtained by excitation at 340 nm to that by excitation at 380 nm. PAR-2 AP: agonist peptide of PAR-2. PAR-2 RP: peptide with the reversed sequence of PAR-2 AP. WCE: whole mite culture extract. Inhibitors: combination of AEBSF and E-64. Data are representative of more than two independent experiments.

(Fig. 5I), and rDer p 1 (Fig. 5J). On treatment with protease inhibitors, WCE (Fig. 5G) and trypsin (Fig. 5D) lost their activity. Prestimulation with WCE (Fig. 5H) or trypsin (Fig. 5E) diminished the subsequent response to PAR-2 AP. The WCE (Fig. 5K) but not rDer f 1 (Fig. 5I) and rDer p 1 (Fig. 5J) diminished the subsequent response to trypsin. The results suggest that WCE and trypsin stimulate keratinocytes through PAR-2 and that this keratinocyte activation is associated with the serine protease (Fig. 5A–H, K). The actions of rDer f 1 and rDer p 1 on keratinocytes are not related to Ca^{2+} mobilization (Fig. 5I,J).

Peptides generated with digestion of a PAR-2 N-terminal peptide by rDer f 1 did not significantly stimulate release of IL-8

Whether rDer f 1 can cleave a synthetic peptide, which contains the activating cleavage site of PAR-2, to generate peptides that promote the release of IL-8 from keratinocytes, was examined (Fig. 6). Peptide #1 corresponding to

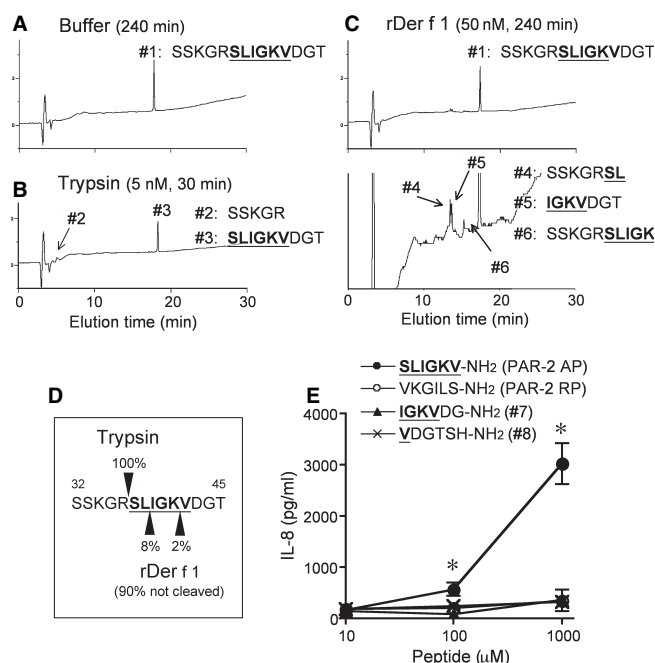


Figure 6. Cleavage analysis of a PAR-2 N-terminal peptide by rDer f 1. (A–C) HPLC for peptides generated by incubation of Peptide #1 corresponding to residues 32–45 in the human PAR-2 sequence with buffer (A), trypsin (B) or rDer f 1 (C). In (C), the vertical scale was changed in the lower panel to visualize small peaks in the upper panel. (D) Sites of cleavage by trypsin and rDer f 1. (E) IL-8 release from primary human keratinocytes stimulated with synthetic peptides putatively exposed by cleavage with rDer f 1. Culture supernatants were recovered at 48 h. PAR-2 AP: agonist peptide of PAR-2. PAR-2 RP: peptide with the reversed sequence of PAR-2 AP. * $P < 0.05$ compared with the negative control (PAR-2 RP) by ANOVA. The sequence of PAR-2 AP is underlined. Data are representative of two independent experiments.

residues 32–45 in the human PAR-2 sequence was incubated with buffer alone (Fig. 6A), trypsin as a control that is known to cleave PAR-2 at the activating site (Fig. 6B), or rDer f 1 (Fig. 6C, upper and lower panels). Cleavage patterns were analyzed by reverse-phase HPLC and the sequences of the generated peptides were determined. Trypsin (5 nM) completely cleaved the peptide at the PAR-2 activation site within 30 min (Fig. 6B,D). On the other hand, cleavage by rDer f 1 (50 nM) was inefficient even at 240 min (Fig. 6C, upper and lower panels) and the cleavage sites were different from that used by trypsin (Fig. 6D). The efficiency of cleavage by rDer f 1 at the two sites was estimated to be approximately 8% and 2% (Fig. 6C, lower panel, and D). As compared with PAR-2AP corresponding to 6 residues after the site of cleavage by trypsin (Fig. 6D,E), two synthesized peptides corresponding to six residues after the site of cleavage by rDer f 1 (Fig. 6D,E, Peptide #7 and #8) exhibited no significant capacity to stimulate the release of IL-8 from keratinocytes (Fig. 6E).

Discussion

Considerable cysteine protease activity of the major group 1 allergens is detected in mite body and fecal extracts (28, 29). In contrast, serine protease activity is much more abundant in the fecal than body extract (30, 31). The WCE used in this study was a soluble extract of whole culture of the house dust mite *D. farinae*, including fecal pellets and body fragments, and the extraction was performed under mild conditions with PBS. We examined the activation of keratinocytes stimulated with this WCE having major serine protease activity and minor cysteine protease activity and compared it with that by recombinant group 1 allergens with cysteine protease activity alone (Fig. 1). The concentrations of WCE (10 µg/ml in Figs 2–4 and 50 µg/ml in Fig. 5) and the recombinant group 1 allergens (100 nM = 2.5 µg/ml in Figs 2–4 and 500 nM = 12.5 µg/ml in Fig. 5) used for stimulation of keratinocytes in the present study are considered relevant to exposure to house dust mite-derived particles because mite fecal pellets are known to contain the group 1 allergen at relatively high concentrations (10 mg/ml) (32). The serine protease activity of WCE induced the release of IL-8, an inflammatory cell-attracting chemokine, and GM-CSF, which has many actions relevant to allergic diseases including dendritic-cell activation (16) (Figs 2, 3A and 4); however, different patterns of mRNA expression for IL-8 and GM-CSF (Fig. 3B) and intracellular Ca^{2+} mobilization (Fig. 5) between WCE and recombinant group 1 allergens, and cleavage analysis of an N-terminal peptide of PAR-2 (Fig. 6) suggested that mite serine and cysteine proteases stimulate keratinocytes, although via different mechanisms, i.e. a PAR-2-dependent mechanism and another mechanism, respectively.

Serine proteases cleave the N-terminal peptide of each PAR and a newly exposed N-terminus, termed a 'tethered' ligand, interacts with residues within the remaining receptor, resulting in activation (33). Synthetic peptides based on the sequence of the tethered ligand can also bind to and activate the receptor. Among the four PARs identified to date, PAR-2 in particular has been implicated in the development of inflammatory reactions including allergies. Mite serine proteases, Der p 3 and Der p 9, activate PAR-2 in an airway epithelial cell line, A549 (13). In the present study, mite-derived serine protease activities within WCE activated keratinocytes to upregulate the expression of IL-8 and GM-CSF at the protein (Figs 2, 3A and 4) and mRNA levels (Fig. 3B) and to induce the intracellular mobilization of Ca^{2+} via the PAR-2 pathway (Fig. 5), similarly to PAR-2 agonists, PAR-2 AP and trypsin. This is the first demonstration that mite-derived serine protease activity activates keratinocytes and offers the first evidence supporting the hypothesis that mite serine proteases contribute to sensitization through the skin and/or the pathogenesis of AD by activating keratinocytes, which was not included in a previous paper (22).

This discrepancy between our results and this paper (22) might arise from differences in the preparation of the mite extract, conditions of cell culture and stimulation, keratinocytes used for experiments, expression levels of the protease inhibitors, and so on.

Some preparations of natural mite group 1 allergens contain traces of serine protease activity because of contamination by mite serine proteases (15, 26); therefore, recombinant mite group 1 allergens rDer f 1 and rDer p 1, which exclusively exhibit cysteine protease activity (24, 26), are useful for accurate analysis of the pathogenicity of their cysteine protease activity (3, 20, 21), which are distinct from those of mite serine proteases. rDer f 1 had much less of an effect on the mRNA expression than WCE (Fig. 3B) and, in the Ca^{2+} mobilization assay, rDer f 1 (Fig. 5I) and rDer p 1 (Fig. 5J) did not activate keratinocytes and did not diminish the subsequent response of keratinocytes to trypsin. rDer f 1 inefficiently cleaved an N-terminal peptide of PAR-2 at different sites from the activation-cleavage site used by trypsin (Fig. 6C, lower panel, and D), but the 6-residue peptides beginning from the site of cleavage by rDer f 1 (Fig. 6D,E, Peptides #7 and #8) had no capacity to stimulate IL-8 release (Fig. 6E). The results suggest that the cysteine protease activity of mite group 1 allergens stimulates keratinocytes to release IL-8 and GM-CSF, although via a different mechanism from mite serine proteases.

Sun et al. (13) demonstrated that Der p 3 and Der p 9 cleave an N-terminal peptide of PAR-2 at the activation-cleavage site and induce the mobilization of Ca^{2+} via the activation of PAR-2 in a human airway epithelial cell line, A549, and the release of GM-CSF and eotaxin. Recently, Adam et al. (34) demonstrated greater upregulation of IL-8 mRNA expression in A549 cells stimulated with Der p 3 than Der p 1, the mobilization of Ca^{2+} by Der p 3 but not Der p 1 in rat PAR-2-transfected KNRK rat kidney cells, and the cleavage of a synthetic N-terminal peptide of rat PAR-2 at different sites from trypsin by Der p 1. Our results in keratinocytes or using human PAR-2 peptides are consistent with their results in airway epithelial cells (13, 34) or using rat PAR-2 peptides (34).

Skin barrier dysfunction is a major manifestation of atopic dermatitis and its involvement in the pathogenesis has been reported (35–37). Barrier disturbance by genetically determined barrier defects (37–39), that by detergents (40) or proteases allergens (8, 9, 12, 41), or their combination might increase the risk of sensitization to allergens toward IgE production and contribute to the development and/or exacerbation of atopic dermatitis. Mite cysteine and serine proteases degrade epithelial tight junction proteins (8, 12) and we recently demonstrated that cysteine protease activity of rDer f 1 with yeast-derived hyperglycosylation caused a reduction in the barrier function of the skin in nude mice (9). Jeong et al. (41) have reported that cockroach and mite extracts delay epidermal permeability barrier recovery

when topically applied to barrier-disrupted sites on mouse and human skin and that, at least for the cockroach extract, its serine protease activity was responsible for the effect. Skin barrier disruption by genetic or environmental factors is considered to facilitate the interaction between keratinocytes and house dust particles containing mite fecal pellets and body fragments with protease activity (28–31); therefore, our results suggest that mite serine and cysteine proteases activate KCs to upregulate the release of IL-8, GM-CSF, and possibly other molecules, which attract and/or activate inflammatory cells and antigen-presenting cells, and the possible role of mite proteases in the initiation of sensitization through the skin and pathogenesis of atopic dermatitis. Furthermore, mite-derived protease activity could activate inflammatory cells, which were attracted to the mite protease-exposed epidermis. An IgE-independent activation of mast cells and basophils has been reported (42). Cysteine protease activity of Der f 1 and papain, which is homologous to mite group 1 allergens, induces the degranulation of eosinophils (43), and Fujisawa et al. (44) have reported that mite extract induced eosinophils to express IL-9 and this was inhibited by a serine protease-specific inhibitor. Interestingly, the cysteine protease activity of our recombinant mite group 1 allergens crucially commits to the sensi-

zation process in mice (3), and Sokol et al. (45) have demonstrated that cysteine protease activity of papain initiates Th2 sensitization in mice via the activation of basophils.

In summary, we demonstrated the activation of keratinocytes by mite-derived serine protease activity for the first time and compared with that by highly purified recombinant group 1 allergens, which exhibit cysteine protease activity alone. Both mite serine and cysteine proteases stimulated keratinocytes to release more IL-8 and GM-CSF, suggesting that they contribute to the sensitization and/or pathogenesis of atopic dermatitis by activating keratinocytes, although via different mechanisms, i.e. via the activation of PAR-2 and another mechanism, respectively. The importance of keratinocyte activation by mite proteases in atopic dermatitis should be addressed in future studies.

Acknowledgements

The authors thank Hikari Taka for mass spectrometry, Francois Niyonsaba, Noriko Shindo, Hiroko Ushio, and Keiko Maeda for technical advice, Tomoko Tokura for technical assistance, Michiyo Matsumoto and Emiko Kawasaki for secretarial assistance, and Masafumi Yaguchi for comments and encouragement.

References

1. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002;**129**:1–18.
2. Chapman MD, Wunschmann S, Pomes A. Proteases as Th2 adjuvants. *Curr Allergy Asthma Rep* 2007;**7**:363–367.
3. Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H et al. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p1 to sensitization toward IgE and IgG responses. *J Immunol* 2006;**177**:1609–1617.
4. Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007;**447**:92–96.
5. Traidl-Hoffmann C, Mariani V, Hochrein H, Karg K, Wagner H, Ring J et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J Exp Med* 2005;**201**:627–636.
6. Boldogh I, Bacsí A, Choudhury BK, Dharajiyá N, Alam R, Hazra TK et al. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J Clin Invest* 2005;**115**:2169–2179.
7. Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999;**190**:1897–1902.
8. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999;**104**:123–133.
9. Nakamura T, Hirasawa Y, Takai T, Mitsuishi K, Okuda M, Kato T et al. Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der f 1. *J Invest Dermatol* 2006;**126**:2719–2723.
10. Shakib F, Schulz O, Sewell H. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 1998;**19**:313–316.
11. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998;**161**:3645–3651.
12. Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ et al. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2001;**31**:279–294.
13. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001;**167**:1014–1021.
14. Seto T, Takai T, Ebihara N, Matsuoka H, Wang XL, Ishii A et al. SLPI prevents cytokine release in mite protease-exposed conjunctival epithelial cells. *Biochem Biophys Res Commun* 2009;**379**:681–685.

15. Brown A, Farmer K, MacDonald L, Kalsheker N, Pritchard D, Haslett C et al. House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am J Respir Cell Mol Biol* 2003;**29**:381–389.
16. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;**8**:193–204.
17. Kinoshita H, Takai T, Le TA, Kamijo S, Wang XL, Ushio H et al. Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA. *J Allergy Clin Immunol* 2009;**123**:179–186.
18. Tan BB, Weald D, Strickland I, Friedmann PS. Double-blind controlled trial of effect of housedust-mite allergen avoidance on atopic dermatitis. *Lancet* 1996;**347**:15–18.
19. Yasueda H, Saito A, Nishioka K, Kutsuwada K, Akiyama K. Measurement of *Dermatophagoides* mite allergens on bedding and human skin surfaces. *Clin Exp Allergy* 2003;**33**:1654–1658.
20. Kato T, Takai T, Mitsuishi K, Okumura K, Ogawa H. Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1: biochemical skin barrier against mite cysteine proteases. *J Allergy Clin Immunol* 2005;**116**:169–176.
21. Ogawa T, Takai T, Kato T, Kikuchi T, Niyonsaba F, Ikeda S et al. Upregulation of release of granulocyte-macrophage colony-stimulating factor from keratinocytes stimulated with cysteine protease activity of recombinant major mite allergens, Der f 1 and Der p 1. *Int Arch Allergy Immunol* 2008;**146**:27–35.
22. Mascia F, Mariani V, Giannetti A, Girolomoni G, Pastore S. House dust mite allergen exerts no direct proinflammatory effects on human keratinocytes. *J Allergy Clin Immunol* 2002;**109**:532–538.
23. Matsuoka H, Maki N, Yoshida S, Arai M, Wang J, Oikawa Y et al. A mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. *Allergy* 2003;**58**:139–145.
24. Takai T, Kato T, Yasueda H, Okumura K, Ogawa H. Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: major conformational IgE epitopes blocked by prodomains. *J Allergy Clin Immunol* 2005;**115**:555–563.
25. Takahashi K, Takai T, Yasuhara T, Yokota T, Okumura Y. Effects of site-directed mutagenesis in the cysteine residues and the N-glycosylation motif in recombinant Der f 1 on secretion and protease activity. *Int Arch Allergy Immunol* 2001;**124**:454–460.
26. Takai T, Kato T, Sakata Y, Yasueda H, Izuhara K, Okumura K et al. Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity. *Biochem Biophys Res Commun* 2005;**328**:944–952.
27. Schechter NM, Brass LF, Lavker RM, Jensen PJ. Reaction of mast cell proteases trypsin and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J Cell Physiol* 1998;**176**:365–373.
28. Ino Y, Ando T, Haida M, Nakamura K, Iwaki M, Okudaira H et al. Characterization of the proteases in the crude mite extract. *Int Arch Allergy Appl Immunol* 1989;**89**:321–326.
29. Ando T, Ino Y, Haida M, Honma R, Maeda H, Yamakawa H et al. Isolation of cysteine protease in the crude mite extract, *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* 1991;**96**:199–205.
30. Ando T, Homma R, Ino Y, Ito G, Miyahara A, Yanagihara T et al. Trypsin-like protease of mites: purification and characterization of trypsin-like protease from mite faecal extract *Dermatophagoides farinae*. Relationship between trypsin-like protease and Der f III. *Clin Exp Allergy* 1993;**23**:777–784.
31. Stewart GA, Kollinger MR, King CM, Thompson PJ. A comparative study of three serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae*. *Allergy* 1994;**49**:553–560.
32. Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. *Nature* 1981;**289**:592–593.
33. Shpacovitch V, Feld M, Bunnett NW, Steinhoff M. Protease-activated receptors: novel PARTners in innate immunity. *Trends Immunol* 2007;**28**:541–550.
34. Adam E, Hansen KK, Astudillo Fernandez O, Coulon L, Bex F, Duhant X et al. The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. *J Biol Chem* 2006;**281**:6910–6923.
35. Ogawa H, Yoshiike T. A speculative view of atopic dermatitis: barrier dysfunction in pathogenesis. *J Dermatol Sci* 1993;**5**:197–204.
36. Cork MJ, Robinson DA, Vasilopoulos Y, Ferguson A, Moustafa M, MacGowan A et al. New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene-environment interactions. *J Allergy Clin Immunol* 2006;**118**:3–21.
37. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006;**38**:441–446.
38. Walley AJ, Chavanas S, Moffatt MF, Esnouf RM, Ubhi B, Lawrence R et al. Gene polymorphism in Netherton and common atopic disease. *Nat Genet* 2001;**29**:175–178.
39. Vasilopoulos Y, Cork MJ, Teare D, Marinou I, Ward SJ, Duff GW et al. A nonsynonymous substitution of cystatin A, a cysteine protease inhibitor of house dust mite protease, leads to decreased mRNA stability and shows a significant association with atopic dermatitis. *Allergy* 2007;**62**:514–519.
40. Okuda M, Yoshiike T, Ogawa H. Detergent-induced epidermal barrier dysfunction and its prevention. *J Dermatol Sci* 2002;**30**:173–179.
41. Jeong SK, Kim HJ, Youm JK, Ahn SK, Choi EH, Sohn MH et al. Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J Invest Dermatol* 2008;**128**:1930–1939.
42. Machado DC, Horton D, Harrop R, Peachell PT, Helm BA. Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *Eur J Immunol* 1996;**26**:2972–2980.
43. Miike S, Kita H. Human eosinophils are activated by cysteine proteases and release inflammatory mediators. *J Allergy Clin Immunol* 2003;**111**:704–713.
44. Fujisawa T, Katsumata H, Kato Y. House dust mite extract induces interleukin-9 expression in human eosinophils. *Allergol Int* 2008;**57**:141–146.
45. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008;**9**:310–318.