

Upregulation of the 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

Takasuke Ogawa^{a, b} Toshiro Takai^a Takeshi Kato^a Yuko Kikuchi^{a, b}
François Niyonsaba^a Shigaku Ikeda^b Ko Okumura^{a, c} Hideoki Ogawa^{a, b}

^aAtopy (Allergy) Research Center, and Departments of ^bDermatology and ^cImmunology, Juntendo University School of Medicine, Tokyo, Japan

Key Words

Keratinocytes • Cystatin A • Granulocyte-macrophage colony-stimulating factor • Recombinant allergens • House dust mite • Der f 1 • Der p 1 • Cysteine protease

Abstract

Background: Although exposure to mite allergens is an important risk factor for the production of IgE and is associated with various allergic diseases, there has been uncertainty as to the route of exposure by which sensitization occurs. Cystatin A is a skin-derived dominant inhibitor against proteolytic activity of major mite allergens, Der f 1 and Der p 1, and blocks the upregulation of IL-8 release from human keratinocytes stimulated with the allergens. We analyzed whether the stimulation of keratinocytes with the allergens upregulates the release of granulocyte-macrophage colony-stimulating factor (GM-CSF), which has many actions relevant to allergic diseases including atopic dermatitis, and if so, whether cystatin A can block this process. **Methods:** Normal human keratinocytes and the human keratinocyte cell line HaCaT were stimulated with recombinant group 1 allergens in the absence or presence of cystatin A. **Results:** Stimulation with the recombinant allergens upregulated the release of

GM-CSF from normal human keratinocytes in a culture with high calcium concentration and HaCaT cells, which could be inhibited by the addition of cystatin A. The allergens exhibiting proteolytic activity did not digest cystatin A. Proteolytic activity of recombinant Der f 1 was partially regenerated after incubation with keratinocytes even without preactivation by L-cysteine. **Conclusion:** Proteolytic activity of recombinant Der f 1 and Der p 1 upregulates GM-CSF and IL-8 release from keratinocytes in vitro, suggesting possible contributions to sensitization through the skin and the perpetuation of atopic dermatitis, as well as a homeostatic role for cystatin A against inflammation of the skin.

Copyright © 2007 S. Karger AG, Basel

Introduction

Exposure to house dust mite allergens is an important risk factor for the production of IgE and is associated with allergic diseases such as asthma, rhinitis and atopic dermatitis (AD) [1–3]. House dust mite group 1 allergens, Der f 1 from *Dermatophagoides farinae* and Der p 1 from *Dermatophagoides pteronyssinus*, are major allergens belonging to the papain-like cysteine protease family [4].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1018–2438/08/1461–0027\$24.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Toshiro Takai
Atopy (Allergy) Research Center, Juntendo University School of Medicine
2-1-1 Hongo, Bunkyo-ku
Tokyo 113-8421 (Japan)
Tel. +81 3 5802 1591, Fax +81 3 3813 5512, E-Mail t-takai@med.juntendo.ac.jp

Their proteolytic activity has been suggested to be involved in the pathogenesis of allergies by facilitating the passage of their own and other allergens across the epithelium and skin [5–7], cleaving and/or interacting with cell surface molecules and intrinsic protease inhibitors and modulating the functions of various cells [8–15]. The cysteine protease activity is inactivated by oxidation of the catalytic thiol group of mite group 1 allergens during storage or purification but can be regenerated by treatment with reducing reagents such as dithiothreitol (DTT), L-cysteine, glutathione and others. Although Herbert et al. [5] speculated that Der p 1 could be activated by reduction with glutathione present in airway tissue on the basis of their *in vitro* results, where and how cysteine protease activity of the mite group 1 allergens can be activated *in vivo* is unclear.

Using a yeast expression system, we succeeded in the preparation of recombinant forms of Der p 1 (rDer p1) and Der f 1 (rDer f 1), which are similar to their natural counterparts in allergenicity and show a similar substrate specificity for cysteine protease activity to the natural types [16–19]. They show cysteine but no serine protease activity, which was often reported to be detected in natural types purified from mite culture extract possibly due to contamination of mite-derived serine proteases [18]. Recombinant forms without yeast-derived hyperglycosylation exhibit similar molecular sizes and secondary structures to their natural types [17, 19]. Like the natural Der p 1, they also cleave human CD23, CD25 and α_1 -antitrypsin and elicit IgE production in mice [11, 20, 21], and the stimulation of human keratinocytes upregulates the release of IL-8 [15]. The proteolytic activity of rDer f 1 with yeast-derived hyperglycosylation caused a reduction in the barrier function of the skin in nude mice [7].

Cystatin A, which is also called 'stefin A', is a skin-derived dominant inhibitor against the cysteine protease activity of Der p 1 and Der f 1 and blocks the upregulation of IL-8 release from keratinocytes stimulated with rDer p 1 and rDer f 1 [15]. Very recently, a significant association of a cystatin A genotype with AD has been reported [22]. Thus, a system including activation and suppression of keratinocyte response against mite cysteine proteases exists in the skin. Analysis of interactions between environmental and genetic factors such as those between protease allergens and their endogenous inhibitors is important to elucidate the pathogenesis of allergic diseases and to prevent their development [23–25].

In airway epithelial cells, cysteine protease activity of Der p 1 has been reported to upregulate the release of granulocyte-macrophage colony-stimulating factor

(GM-CSF) along with IL-8 [12]. GM-CSF has many actions relevant to allergic inflammation including AD [26, 27]. In this study, we analyzed whether the stimulation of keratinocytes with the proteolytic activity of rDer f 1 and rDer p 1 upregulates the release of GM-CSF, and if so, whether cystatin A can block this process.

Materials and Methods

Der f 1, Der p 1 and Cystatin A

rDer f 1 and rDer p 1 without yeast-derived hyperglycosylation (Der f 1-N53Q and Der p 1-N52Q) were prepared as described previously [17, 19, 28]. Recombinant human cystatin A was purchased from R&D Systems (Minneapolis, Minn., USA). Human sweat-derived cystatin A was purified as described [15].

Stimulation of Keratinocytes

Normal human keratinocytes (Cascade Biologics, Portland, Oreg., USA) were cultured in HuMedia-KG2 (0.15 mM CaCl₂) or EpiLife-KG2 (0.06 mM CaCl₂; Kurabo Industries, Osaka, Japan) supplemented with 0.1 ng/ml epidermal growth factor, 10 μ g/ml insulin, 0.5 μ g/ml hydrocortisone, 50 μ g/ml gentamycin, 50 ng/ml amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Cells of the human keratinocyte cell line HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, Mo., USA) supplemented with 10% FCS and antibiotics. They were stimulated with rDer f 1, rDer p 1 and an agonist peptide of the human protease-activated receptor 2 (PAR-2; Bachem, Bubendorf, Switzerland) in basal media without the supplements, as previously described [15]. The cells were incubated for 24 or 48 h, and the supernatants were collected and subjected to ELISA to measure released cytokines including GM-CSF and IL-8 using kits (DuoSet; R&D Systems).

Proteolytic Activity

Proteolytic activity was measured as described previously [18], with some modifications, as indicated in the figure legends. Time courses of digestion of a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA (Peptide Institute, Osaka, Japan), were monitored.

SDS-PAGE Analysis

The recombinant group 1 allergens were activated with L-cysteine as described previously [20]. Activated rDer f 1 and rDer p 1 at 400 nM were added to 100 nM human sweat cystatin A or 200 nM human α_1 -antitrypsin as a positive control in reaction buffer composed of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. Unactivated rDer f 1 and rDer p 1 were used as controls. After incubation for 2 h at 37°C, the samples were subjected to SDS-PAGE.

Statistical Analysis

Group data were statistically analyzed with Prism version 4.0 (GraphPad, San Diego, Calif., USA). The unpaired Student's *t* test (two-tailed) or the Tukey post hoc test followed by a one-way ANOVA was used to evaluate the significance of differences. A *p* value <0.05 was regarded as statistically significant.

Results

Release of IL-8 from Keratinocytes Stimulated with rDer f 1

The dose and time dependency of the release of IL-8 from keratinocytes stimulated with activated rDer f 1 was analyzed (fig. 1). Cysteine protease activity of rDer f 1 was regenerated by pretreatment with L-cysteine. The release from HaCaT cells and primary cultured normal human keratinocytes was upregulated by 100 nM of rDer f 1 in the supernatants collected at 24 and 48 h after stimulation. The concentration of IL-8 released was higher at 48 h than at 24 h.

Release of GM-CSF and IL-8 from Keratinocytes Stimulated with rDer p 1 and rDer f 1 and Its Inhibition by Cystatin A

Recombinant cystatin A dose-dependently inhibited the protease activity of rDer p 1 and rDer f 1 (fig. 2a). HaCaT cells released more GM-CSF and IL-8 on stimulation with activated rDer p 1 and rDer f 1 at 100 nM after 48 h than unstimulated cells, and the addition of an equimolar amount of cystatin A blocked this process (fig. 2b). At a low calcium concentration, the release of IL-8 from normal human keratinocytes was upregulated on stimulation with rDer p 1 and rDer f 1 at 100 or 200 nM at 48 h, inhibited by the addition of cystatin A in an equimolar amount, while the release of GM-CSF was not upregulated (fig. 2c). At a high calcium concentration, release of both GM-CSF and IL-8 from normal human keratinocytes was upregulated, blocked by the addition of cystatin A (fig. 2d). The release of GM-CSF upregulated by the stimulation with rDer p 1 and rDer f 1 was greater in normal human keratinocytes than in HaCaT cells (fig. 2b, d, left panels). The values of GM-CSF and IL-8 on stimulation with an agonist peptide of PAR-2 as a positive control were not affected by the addition of cystatin A, indicating the blocking effect by cystatin A to be specific to the proteolytic activity of rDer p 1 and rDer f 1.

Cystatin A Was Not Digested by the Proteolytic Activity of rDer p 1 and rDer f 1

The stability of cystatin A on exposure to the proteolytic activity of rDer p 1 and rDer f 1 was evaluated by SDS-PAGE. Sweat-derived cystatin A was stable even though it interacted with active rDer p 1 or rDer f 1 (fig. 3). Under the same conditions in the presence of L-cysteine, they cleaved α_1 -antitrypsin, as reported previously [20].

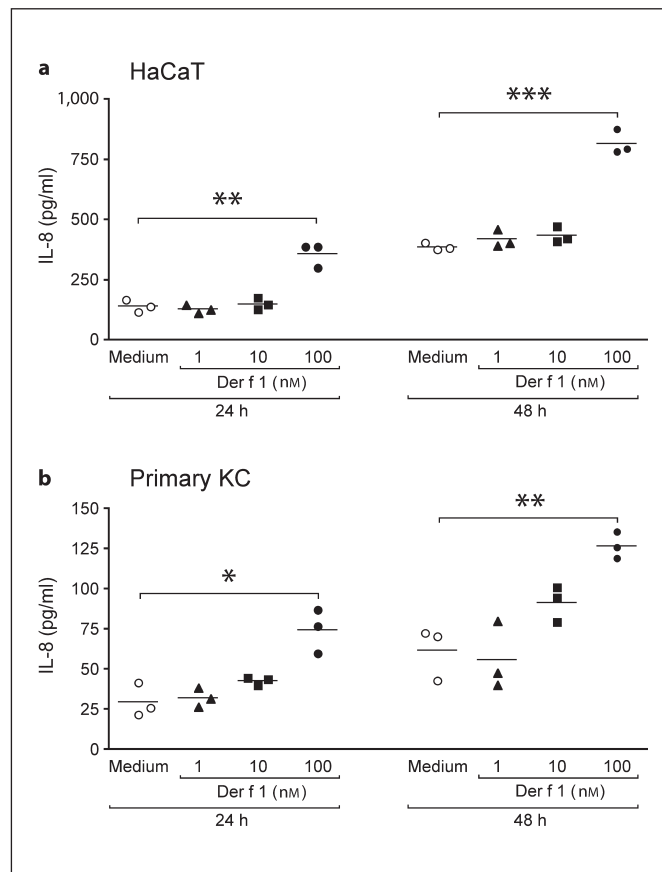
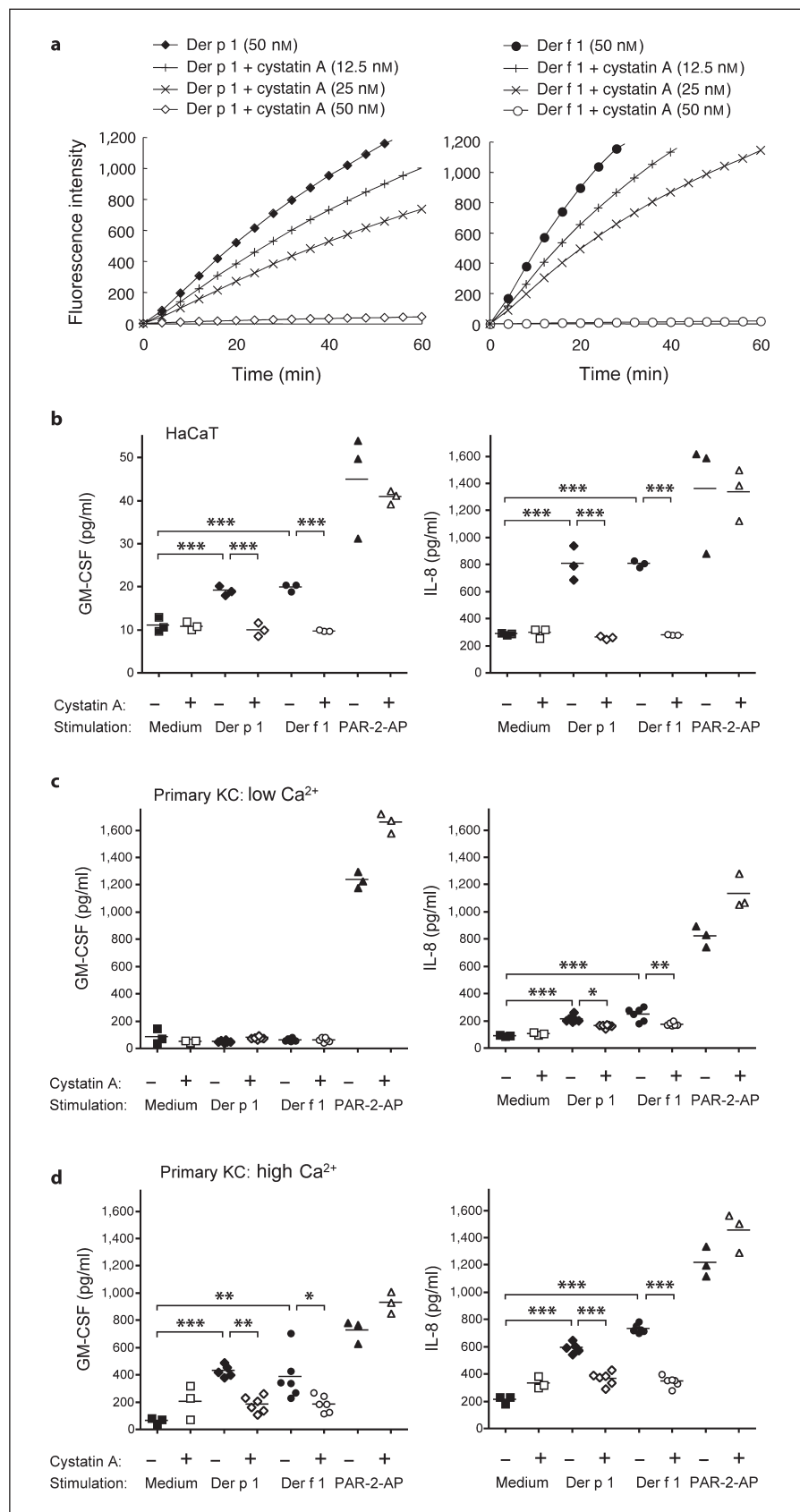


Fig. 1. Dose and time dependency of IL-8 release from keratinocytes stimulated with rDer f 1. **a** Human keratinocyte cell line HaCaT. **b** Primary cultured normal human keratinocytes (KC). Cysteine protease activity of rDer f 1 was activated with L-cysteine prior to stimulation of the cells. Basal media of DMEM and HuMedia-KG2 were used for the stimulation of HaCaT and normal human keratinocytes, respectively. The culture supernatants collected 24 or 48 h after the beginning of the stimulation were assayed. Each point corresponds to the value for each well. Results for 3 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the control (medium) by unpaired t test (two-tailed).

rDer f 1 with or without Pretreatment with L-Cysteine Upregulated the Release of IL-8 and GM-CSF

Whether regeneration of cysteine protease activity by pretreatment with L-cysteine prior to the stimulation of keratinocytes is essential to the upregulation of the release of the proinflammatory cytokines was analyzed. With or without the pretreatment with L-cysteine, rDer f 1 upregulated the release of IL-8 (fig. 4) and GM-CSF (fig. 5c) from normal human keratinocytes.

Fig. 2. Release of GM-CSF and IL-8 from keratinocytes stimulated with rDer p 1 and rDer f 1 and its inhibition by cystatin A. **a** Inhibition of proteolytic activity of rDer p 1 and rDer f 1 by recombinant cystatin A. rDer p 1 and rDer f 1 were activated with DTT, incubated with cystatin A and subjected to the assay in a reaction buffer composed of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. Time courses of digestion of a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA, were measured on a fluorometer (Fluoroskan Ascent; Labsystems, Vantaa, Finland). Final concentrations of the allergens, DTT and the substrate were 50 nM, 1 mM and 0.1 mM, respectively. Final concentrations of cystatin A are indicated (12.5, 25 and 50 nM). **b–d** Release of GM-CSF and IL-8 from HaCaT cells and primary cultured normal human keratinocytes (KC). rDer p 1 and rDer f 1 were activated with L-cysteine prior to stimulation of the cells. Basal medium of DMEM was used for the stimulation of HaCaT (**b**). Basal media of HuMedia-KG2 containing 0.15 mM (**c**) and 1.35 mM CaCl_2 (**d**) were used for the stimulation of normal keratinocytes. Final concentrations of the allergens and recombinant cystatin A were 100 or 200 nM. The culture supernatants collected 48 h after the beginning of stimulation were assayed. Each point corresponds to the value for each well. Results for 3 or 6 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between cells stimulated with allergens (Der p 1 or Der f 1) and those unstimulated (medium) and between the absence (–) and presence of cystatin A (+) by the Tukey post hoc test followed by one-way ANOVA (two-tailed). PAR-2-AP = Agonist peptide of PAR-2 (100 μM for HaCaT and 500 μM for normal keratinocytes).



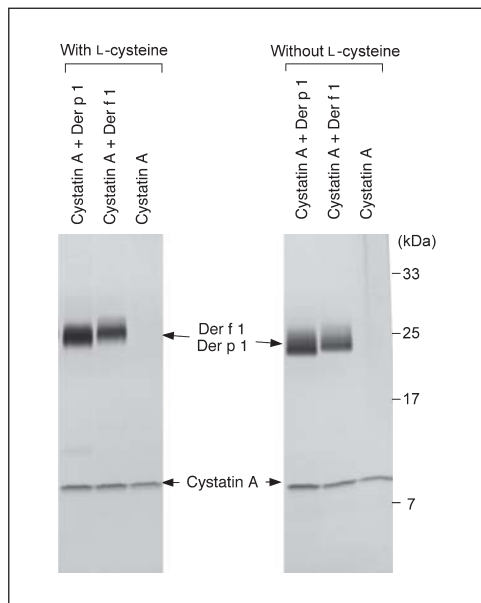


Fig. 3. Cystatin A was not digested by rDer p 1 and rDer f 1. The allergens were incubated in a buffer with or without L-cysteine prior to the reaction. Bands for rDer p 1, rDer f 1 and sweat-derived cystatin A are indicated by arrows. The gel after SDS-PAGE under nonreducing conditions was stained with silver (Silver Stain II kit; Wako, Osaka, Japan).

Proteolytic Activity of rDer f 1 in Culture Media before and after Incubation with Keratinocytes

Just after dilution in the basal media, unactivated rDer f 1 showed almost no proteolytic activity, and rDer f 1 activated with L-cysteine showed lower activity compared with rDer f 1, which was kept active by DTT during the protease assay (fig. 5a).

Culture supernatants recovered 36 h after the beginning of the stimulation of normal keratinocytes showed proteolytic activity (fig. 5b, upper panel), although the activity was lower (1/100 or less) compared with those kept active by DTT during the protease assay (fig. 5b, lower panel). The activity was similar between unactivated rDer f 1 and rDer f 1 activated with L-cysteine prior to the stimulation (fig. 5b, upper panel) and was eliminated by addition of fresh cystatin A in the protease assay (unpublished data). Keratinocytes were detached from the bottom of the culture plate at 400 nM of rDer f 1 with or without pretreatment with L-cysteine but not at 100 nM (unpublished data). Addition of cystatin A to the stimulation kept the proteolytic activity inactive (fig. 5b, upper panel, cystatin A+), even in the presence of DTT during the protease assay (fig. 5b, lower panel, cystatin A+), and inhibited the upregulation of GM-CSF release (fig. 5c, cystatin A+).

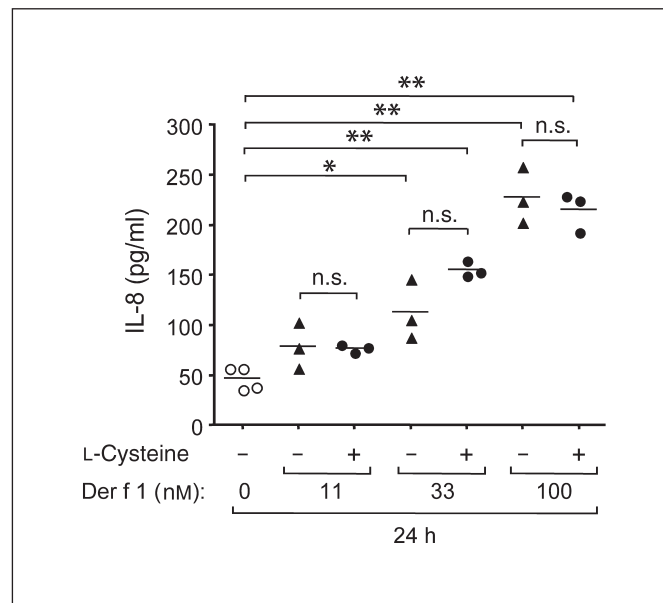
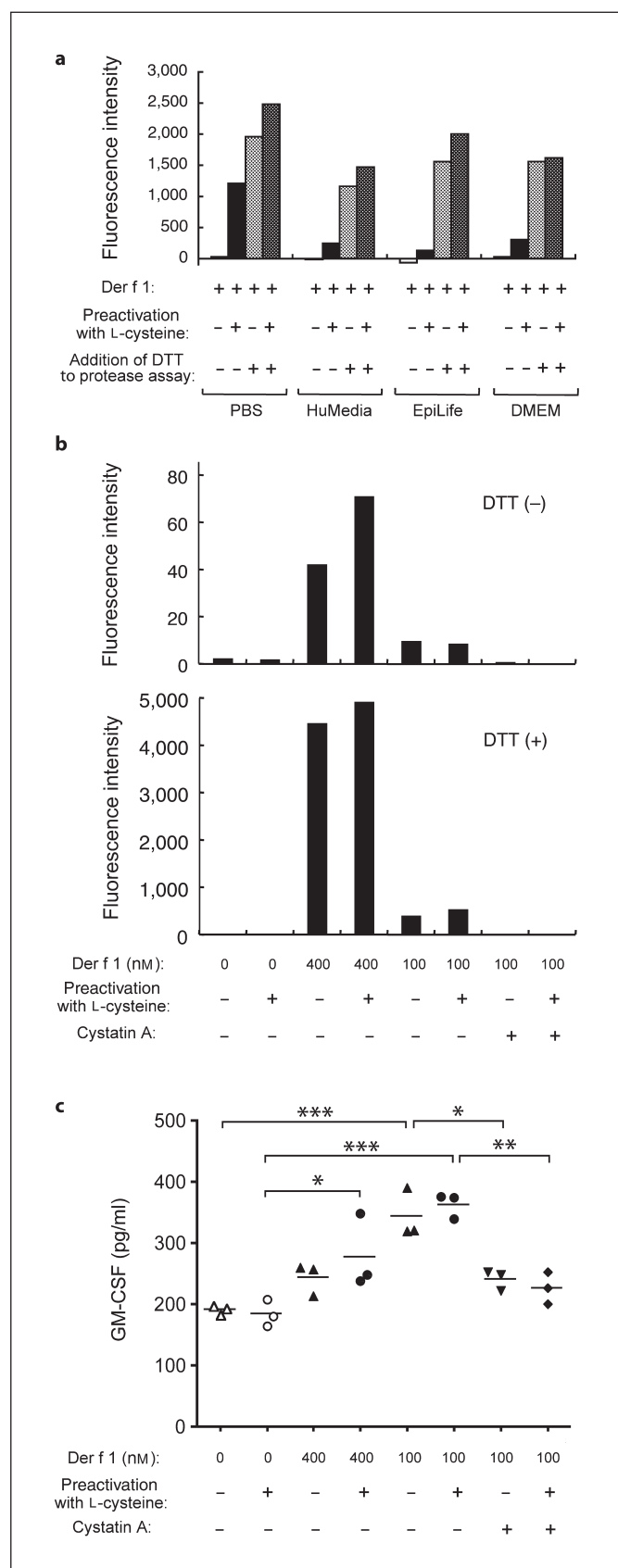


Fig. 4. rDer f 1 with or without preactivation with L-cysteine up-regulated IL-8 release from primary cultured normal human keratinocytes. rDer f 1 were activated with L-cysteine (+) or unactivated (-) prior to stimulation of the cells. Basal medium of HuMedia-KG2 was used for the stimulation. The culture supernatants collected 24 h after the beginning of the stimulation were assayed. Final concentrations of rDer f 1 were indicated. Each point corresponds to the value for each well. Results for 3 or 4 wells are shown. Bars indicate means. * $p < 0.01$ and ** $p < 0.001$ compared with the negative control by the Tukey post hoc test followed by one-way ANOVA. n.s. = Not significantly different ($p > 0.05$) between groups stimulated with rDer f 1 with and without pretreatment with L-cysteine compared by unpaired t test (two-tailed).

Discussion

Although exposure to mite allergens is an important risk factor for the production of IgE and is associated with various allergic diseases, there has been uncertainty as to the route of exposure by which sensitization occurs. We demonstrated that stimulation of keratinocytes with the proteolytic activity of rDer f 1 and rDer p 1 upregulates the release of GM-CSF along with IL-8 (fig. 1, 2, 4, 5c), and cystatin A, which is the skin-derived dominant and stable inhibitor against the proteolytic activity of the allergens (fig. 2a, 3, 5b), can block this process (fig. 2, 5c). rDer f 1 with or without pretreatment with L-cysteine up-regulated the release of proinflammatory cytokines (fig. 4, 5c), and cysteine protease activity of unactivated rDer f 1 was partially regenerated after incubation with keratinocytes (fig. 5b). These results suggest the importance of the proteolytic activity of mite group 1 allergens



in sensitization through the skin, a homeostatic role for cystatin A, and that the catalytic thiol group of the cysteine protease allergens, which was oxidized during storage or purification, could be regenerated by interaction with the cells or components in the culture supernatant.

GM-CSF has many actions relevant to allergic diseases such as AD. A recent hypothesis is that lesional up-regulation of GM-CSF contributes to the maintenance of the chronic inflammatory process in AD primarily by enhancing the antigen-processing capacity of Langerhans and dendritic cells [26]. We have reported that the proteolytic activity of rDer f 1 with yeast-derived hyperglycosylation causes a reduction in barrier function of the skin in nude mice [7]. Taken together, the cysteine protease activity of the mite group 1 allergens might be involved in sensitization through the skin by reducing the barrier function of the skin and providing a portal of entry for allergens and irritants, and by subsequently stimulating keratinocytes to upregulate the release of GM-

Fig. 5. Proteolytic activity of rDer f 1 in the cell culture media before and after incubation with primary cultured normal human keratinocytes. **a** Proteolytic activity of rDer f 1 in fresh basal media. The reaction was started with diluting rDer f 1 unactivated or activated with 5 mM L-cysteine in phosphate-buffered saline (PBS, pH 7.4) or in the basal media (HuMedia-KG2, EpiLife-KG2 and DMEM) containing a fluorogenic substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA, with or without DTT (1/10 dilution). Time courses of digestion of the substrate were measured on a fluorometer (SpectraMAX GeminiEM; Molecular Devices, Sunnyvale, Calif., USA). Fluorescence intensities at 60 min are shown. Final concentrations of rDer f 1, L-cysteine, DTT and the substrate in the protease assay were 100 nM, 0.5, 1 and 0.1 mM, respectively. **b** Proteolytic activity within culture supernatants 36 h after the beginning of the stimulation of normal keratinocytes. The cells were stimulated with rDer f 1 unactivated or activated with L-cysteine in the absence or presence of recombinant cystatin A. Basal medium of EpiLife-KG2 was used for the stimulation. The reaction was started with diluting culture supernatants in PBS containing the substrate with or without DTT (1/2 dilution). Fluorescence intensities at 60 min are shown. The vertical scale is different between the upper and lower panels. Concentrations of rDer f 1 in the stimulation are indicated (400 and 100 nM) and correspond to 200 and 50 nM in the protease assay, respectively. The concentration of cystatin A in the stimulation was 200 nM. Concentrations of DTT and the substrate in the protease assay were 1 and 0.05 mM, respectively. **c** GM-CSF release from normal keratinocytes. The culture supernatants collected 48 h after the beginning of the stimulation were assayed. Each point corresponds to the value for each well. Results for 3 wells are shown. Bars indicate means. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with controls by the Tukey post hoc test followed by one-way ANOVA.

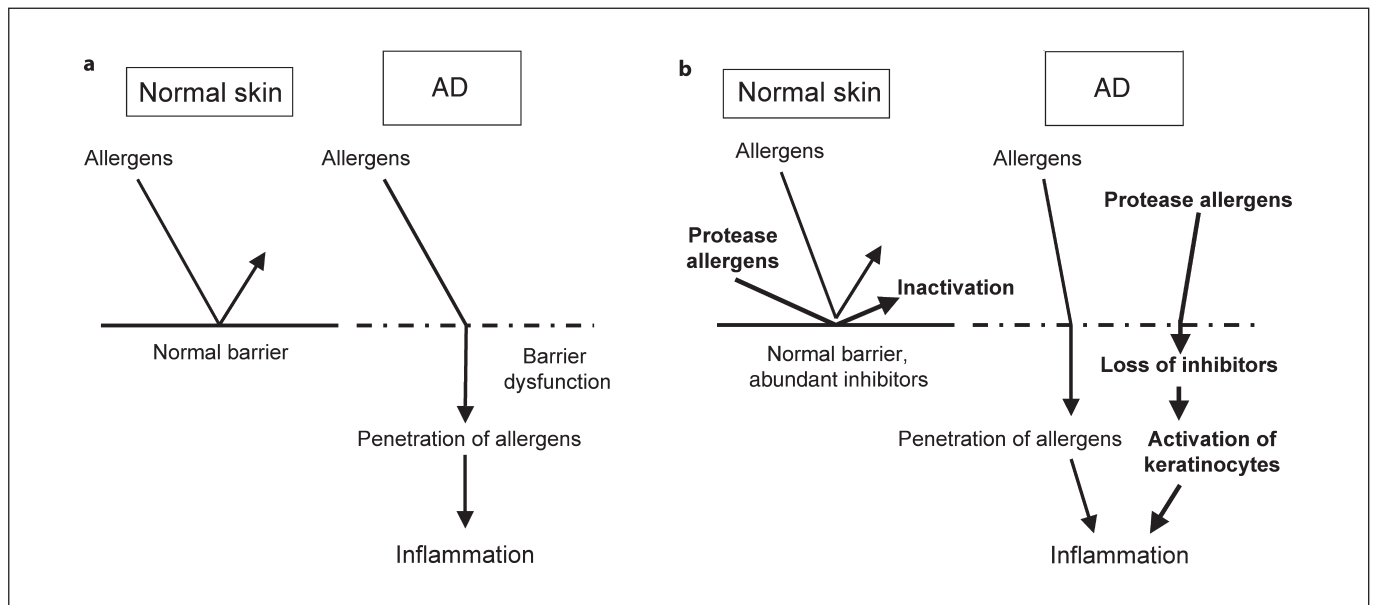


Fig. 6. Physical and biochemical barrier function of the skin. **a** Conventional concept of the 'physical' barrier function of the skin. **b** Protease inhibitors in the skin function as a 'biochemical' barrier against environmental protease allergens.

CSF and IL-8, which could contribute to the initiation and perpetuation of cutaneous inflammation in the microenvironment. Calcium concentration, which is an important factor for differentiation of keratinocytes, seems important in the response of keratinocytes (fig. 2c, d); however, the release of GM-CSF was upregulated at a low calcium concentration in some experiments (fig. 5c and unpublished observations), suggesting that conditions such as cell density and stage of differentiation affect the susceptibility of the cells to stimulation.

Where and how the cysteine protease activity of the mite group 1 allergens can be activated *in vivo* is still unclear, although Herbert et al. [5] speculated that Der p 1 could be activated by reduction with glutathione present in airway tissue on the basis of their *in vitro* results. Interestingly, unactivated rDer f 1 could upregulate the release of proinflammatory cytokines (fig. 4, 5c). Its cysteine protease activity was partially regenerated after incubation with keratinocytes (fig. 5b) and capable to cause cell detachment at 400 nM of rDer f 1 (unpublished data). Cystatin A inhibited the upregulation of GM-CSF release from keratinocytes stimulated with the unactivated rDer f 1 (fig. 5c). These results suggest that oxidized catalytic cysteine residue of the mite group 1 allergens could be reduced by interaction with the cells or components in the culture supernatant including cell-derived factors

and that cysteine protease activity regenerated after incubation with the cells stimulates the keratinocytes.

Cystatin A was stable (fig. 3) even though it interacted with active rDer p 1 or rDer f 1 (fig. 2a), which can be explained by the high-affinity binding of this inhibitor to the cysteine protease and by the finding that the N-terminal hook of cystatin family inhibitors binds to part of the substrate-binding cleft of cysteine proteases in a substrate-like manner but then turns away from the active site, preventing proteolytic processing [29]. The stability of cystatin A is considered to contribute to a homeostatic role of cystatin A against skin inflammation. Indeed, the cystatin A content is reported to have decreased in the lesional skin of patients with AD [30], and very recently, Vasilopoulos et al. [22] found a significant association of a cystatin A genotype, in which mRNA of cystatin A is unstable, with AD. Thus, a decrease in cystatin A by non-genetic [30] or genetic factors [22] could increase the risk of sensitization through the skin and the perpetuation of AD. The physical barrier dysfunction is a major manifestation of AD, and its involvement with the pathogenesis has been reported (fig. 6a) [25, 31, 32]. Adding to this conventional concept, protease inhibitors such as cystatin A could function as a biochemical skin barrier to prevent the activation of keratinocytes by environmental protease allergens (fig. 6b).

Analysis of interactions between environmental and genetic factors such as those between protease allergens and their inhibitors is important to elucidate the pathogenesis of allergic diseases and to prevent their development [23–25]. Proteolytic activity of rDer f 1 and rDer p 1 upregulates GM-CSF and IL-8 release from keratinocytes in vitro, suggesting possible contributions to sensitization through the skin and the perpetuation of AD, as well as a homeostatic role for cystatin A against inflammation of the skin. An analysis of the mechanism of stimulation of keratinocytes with Der f 1 and Der p 1 should be addressed in a future study.

Acknowledgements

We thank Michiyo Matsumoto and Emiko Kawasaki (Juntendo University School of Medicine) for their secretarial assistance. This work was supported in part by a Health and Labour Sciences Research Grant for Research on Allergic Diseases and Immunology from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to T.T.).

References

- Platts-Mills TA, Chapman MD: Dust mites: immunology, allergic disease, and environmental control. *J Allergy Clin Immunol* 1987;80:755–775.
- 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.
- Kramer U, Lemmen C, Bartusel E, Link E, Ring J, Behrendt H: Current eczema in children is related to Der f 1 exposure but not to Der p 1 exposure. *Br J Dermatol* 2006;154:99–105.
- 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.
- Herbert CA, King CM, Ring PC, Holgate ST, Stewart GA, Thompson PJ, Robinson C: Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p 1. *Am J Respir Cell Mol Biol* 1995;12:369–378.
- Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C: Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999;104:123–133.
- Nakamura T, Hirasawa Y, Takai T, Mitsuishi K, Okuda M, Kato T, Okumura K, Ikeda S, Ogawa H: 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.
- 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.
- Comoy EE, Pestel J, Duez C, Stewart GA, Vendeville C, Fournier C, Finkelman F, Capron A, Thyphronitis G: The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN- γ . *J Immunol* 1998;160:2456–2462.
- 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.
- Kikuchi Y, Takai T, Kuhara T, Ota M, Kato T, Hatanaka H, Ichikawa S, Tokura T, Akiba H, Mitsuishi K, Ikeda S, Okumura K, Ogawa H: Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization toward IgE and IgG responses. *J Immunol* 2006;177:1609–1617.
- 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.
- Miike S, Kita H: Human eosinophils are activated by cysteine proteases and release inflammatory mediators. *J Allergy Clin Immunol* 2003;111:704–713.
- Grunstein MM, Veler H, Shan X, Larson J, Grunstein JS, Chuang S: Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J Allergy Clin Immunol* 2005;116:94–101.
- 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.
- Yasuhara T, Takai T, Yuuki T, Okudaira H, Okumura Y: Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding. *Clin Exp Allergy* 2001;31:116–124.
- Takai T, Mineki R, Nakazawa T, Takaoka M, Yasueda H, Murayama K, Okumura K, Ogawa H: Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity. *FEBS Lett* 2002;531:265–272.
- Takai T, Kato T, Sakata Y, Yasueda H, Izuhara K, Okumura K, Ogawa H: 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.
- 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.
- Takai T, Kato T, Ota M, Yasueda H, Kuhara T, Okumura K, Ogawa H: Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25, and α_1 -antitrypsin, and in vivo IgE-eliciting activity in mice. *Int Arch Allergy Immunol* 2005;137:194–200.
- Kikuchi Y, Takai T, Ota M, Kato T, Takeda K, Mitsuishi K, Ikeda S, Okumura K, Ogawa H: Application of immunoreaction enhancer solutions to an enzyme-linked immunosorbent assay for antigen-specific IgE in mice immunized with recombinant major mite allergens or ovalbumin. *Int Arch Allergy Immunol* 2006;141:322–330.
- Vasilopoulos Y, Cork MJ, Teare D, Marinou I, Ward SJ, Duff GW, Tazi-Ahnini R: A non-synonymous 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.
- Cork MJ, Robinson DA, Vasilopoulos Y, Ferguson A, Moustafa M, MacGowan A, Duff GW, Ward SJ, Tazi-Ahnini R: New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene-environment interactions. *J Allergy Clin Immunol* 2006;118:3–21.
- Smith PK, Harper JI: Serine proteases, their inhibitors and allergy. *Allergy* 2006;61:1441–1447.

- 25 Hudson TJ: Skin barrier function and allergic risk. *Nat Genet* 2006;38:399–400.
- 26 Esche C, de Benedetto A, Beck LA: Keratinocytes in atopic dermatitis: inflammatory signals. *Curr Allergy Asthma Rep* 2004;4:276–284.
- 27 Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M: Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* 2004;173:6384–6392.
- 28 Takai T, Mizuuchi E, Kikuchi Y, Nagamune T, Okumura K, Ogawa H: Glycosylation of recombinant proforms of major house dust mite allergens Der p 1 and Der f 1 decelerates the speed of maturation. *Int Arch Allergy Immunol* 2006;139:181–187.
- 29 Jenko S, Dolenc I, Guncar G, Dobersek A, Podobnik M, Turk D: Crystal structure of Stefin A in complex with cathepsin H: N-terminal residues of inhibitors can adapt to the active sites of endo- and exopeptidases. *J Mol Biol* 2003;326:875–885.
- 30 Seguchi T, Cui CY, Kusuda S, Takahashi M, Aisu K, Tezuka T: Decreased expression of filaggrin in atopic skin. *Arch Dermatol Res* 1996;288:442–446.
- 31 Ogawa H, Yoshiike T: A speculative view of atopic dermatitis: barrier dysfunction in pathogenesis. *J Dermatol Sci* 1993;5:197–204.
- 32 Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ, O'Regan GM, Watson RM, Cecil JE, Bale SJ, Compton JG, DiGiovanna JJ, Fleckman P, Lewis-Jones S, Arseculeratne G, Sergeant A, Munro CS, El Houate B, McElreavey K, Halkjaer LB, Bisgaard H, Mukhopadhyay S, McLean WH: 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.