

Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1: Biochemical skin barrier against mite cysteine proteases

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Background: Der p 1 and Der f 1 are the most immunodominant allergens produced by house dust mites and are suspected to be involved in the pathogenesis of allergy through their cysteine protease activity. However, stimulation of keratinocytes by these protease allergens and protective systems in the skin against them have not been well investigated.

Objective: We purified and identified the dominant skin-derived inhibitor against the proteolytic activities of these allergens and analyzed its effect on keratinocyte activation.

Methods: Recombinant allergens were used for the experiments. We analyzed whether human sweat inhibits the enzymatic activities of Der p 1 and Der f 1 and used sweat as the skin-derived material to isolate the inhibitor. The inhibitor was purified by means of column chromatography and subsequently identified by means of protein sequencing and immunoblotting. Keratinocytes were stimulated with the allergens in the absence or presence of the inhibitor, and the concentration of secreted IL-8 was measured.

Results: Sweat inhibited the proteolytic activities of Der p 1 and Der f 1. The sweat inhibitor was identified as cystatin A. The stimulation of normal human keratinocytes and the human keratinocyte cell line HaCaT with these protease allergens upregulated IL-8 secretion, and addition of cystatin A blocked this upregulation. Normal human keratinocytes secreted cystatin A into the medium.

Conclusions: The proteolytic activity of Der p 1 and Der f 1 stimulates human keratinocytes *in vitro*. Cystatin A produced by keratinocytes is the dominant biochemical skin barrier that eliminates the enzymatic activity of these mite cysteine proteases and prevents them from stimulating keratinocytes. (J Allergy Clin Immunol 2005;116:169-76.)

Key words: House dust mite, Der p 1, Der f 1, cysteine protease, skin-derived protease inhibitor, cystatin A, keratinocyte, IL-8, atopic dermatitis

House dust mites of *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are the major source of allergens associated with the occurrence of allergic diseases, such as asthma, rhinitis, and atopic dermatitis (AD).¹ The group 1 allergens, Der p 1 and Der f 1, which belong to the papain-like cysteine protease family, abundantly exist in mite fecal pellets and account for more than 50% of the IgE antibodies present against total mite extract.² The cysteine protease activity of Der p 1 facilitates passage of it and other allergens across the bronchial epithelium,^{3,4} the cleavage and/or interaction with intrinsic protease inhibitors,⁵⁻⁷ and the modulation of function of various cells by cleaving cell-surface molecules.^{5,8-10} In mice 2 research groups reported on the enhancement of IgE synthesis¹¹ and induction of T_H2 responses¹² by the proteolytic activity of Der p 1 *in vivo*. Thus evidence suggests a relationship between the proteolytic activity of mite group 1 allergens and the pathogenesis of allergic diseases.

The cysteine protease activity of Der p 1 directly stimulates airway epithelial cells to release cytokines and chemokines and might be involved in the pathogenesis of respiratory allergy.^{10,13} Recently, the squamous cell carcinoma antigen 2 (SCCA2), an inducible protease inhibitor produced by bronchial epithelial cells, was reported to inhibit the proteolytic activity of Der p 1 and Der f 1.⁷ This suggests that a system including activation and suppression of the epithelial cell response against the proteolytic activity of Der p 1 and Der f 1 exists in the respiratory tract. On the other hand, whether Der p 1 and Der f 1 stimulate keratinocytes has not been thoroughly investigated, although house dust mite allergens are important factors of AD,^{14,15} and Der p 1 and Der f 1 are present on the surface of human skin.¹⁶ Although only one report that investigated whether *D pteronyssinus* extract stimulates keratinocytes described that mite extract did not stimulate keratinocyte proinflammatory functions *in vitro*,¹⁷ the potential effect of house dust mite proteases on human skin under pathophysiologic conditions *in vivo* cannot be excluded. We hypothesized that a system including activation and suppression of the keratinocyte response exists in the skin, as well as in the respiratory

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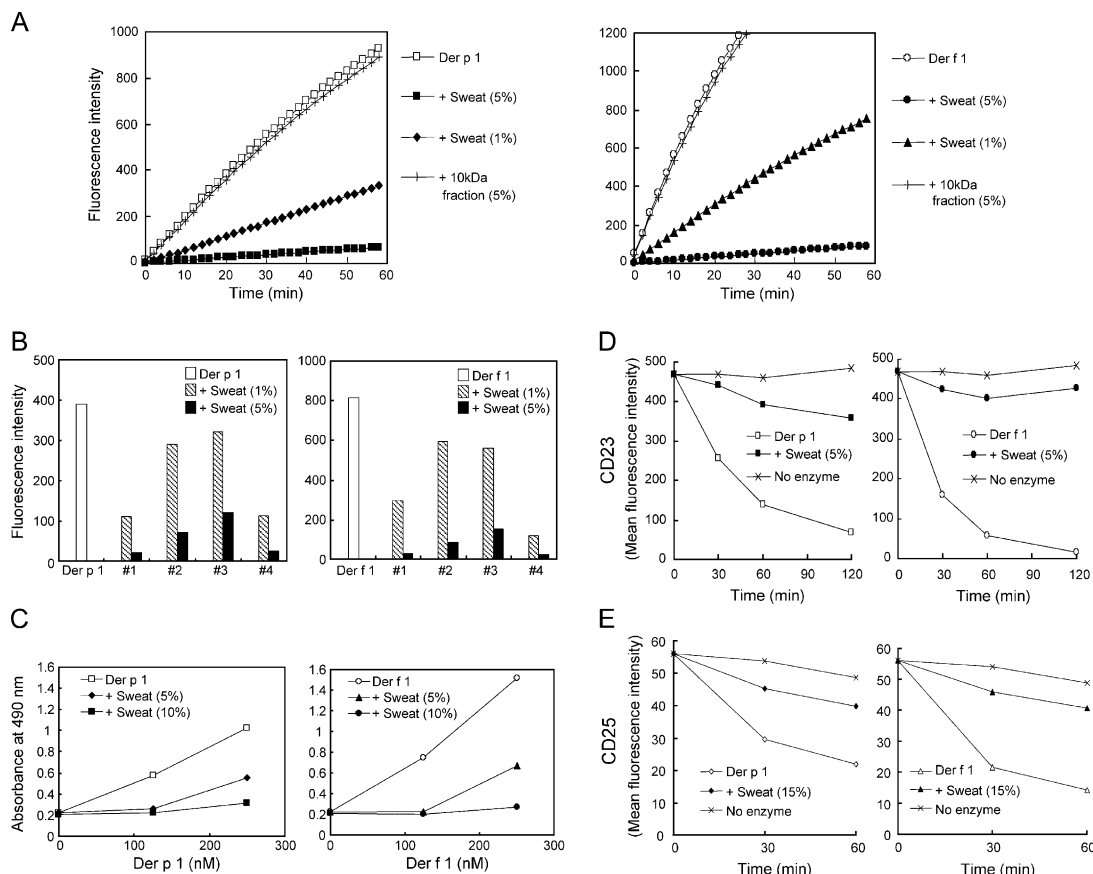


FIG 1. The inhibitory activity of human sweat against the proteolytic activities of Der p 1 and Der f 1. **A** and **B**, The proteolysis of butyloxycarbonyl-Gln-Ala-Arg-MCA. **C**, Azocoll. **D** and **E**, Human CD23 and CD25 on the cell surface. *No enzyme*, Cells were incubated with buffer alone; *+ Sweat*, sweat was added to the allergens before reaction with substrates.

Abbreviations used

AD: Atopic dermatitis
PAR-2: Protease-activated receptor 2
SCCA: Squamous cell carcinoma antigen

tract. We therefore purified and identified the most potent skin-derived inhibitor of the catalytic activity of Der p 1 and Der f 1 and found that it prevented Der p 1 and Der f 1 from stimulating keratinocytes.

METHODS

Sweat

Volunteers cleansed their bodies with soap and rinsed thoroughly with running water. Sweat was then collected from volunteers after sauna sweating, during which they stood in a large plastic bag while sweating, and the sweat dropped into the bottom of the bag, with 10 to 200 mL later recovered. The sweat was afterward sterilized by passing through a 0.45- μ m filter and stored at 4°C.

Preparation of Der p 1 and Der f 1

We used recombinant allergens for the assays consisting of Der p 1-N52Q and Der f 1-N53Q,¹⁸⁻²² which have molecular weights

identical to those of natural allergens, except that Der f 1-WT with yeast-derived hyperglycosylation was used for the assay of the inhibitory activity of samples fractionated by means of column chromatography. Recombinant Der p 1 and Der f 1 are similar to their natural counterparts in their structures and allergenicities,¹⁸ have proteolytic activities,¹⁹⁻²² show similar substrate specificity for the cysteine protease activity of natural Der p 1 and Der f 1,^{19,20} and are free from mite-derived serine proteases.²⁰

Measurement of proteolytic activity with a short synthetic substrate

Proteolytic activity was measured as previously described.^{20,21}

Measurement of proteolytic activity with a collagen substrate

The insoluble diazotized collagen substrate Azocoll (Calbiochem, San Diego, Calif) was used as a protein substrate.¹⁹ After the reaction, the absorbance of the supernatant at 490 nm was measured. Means of the duplicate data were shown.

Cleavage of human CD23 and CD25

The methods used were those described by Schulz et al.^{5,9} Cells at 2×10^6 cells/mL were incubated with 1 μ g/mL or 2 μ g/mL activated enzymes; stained with fluorescein isothiocyanate-labeled mAbs against CD23 or CD25, respectively; and analyzed on a flow cytometer.¹⁹

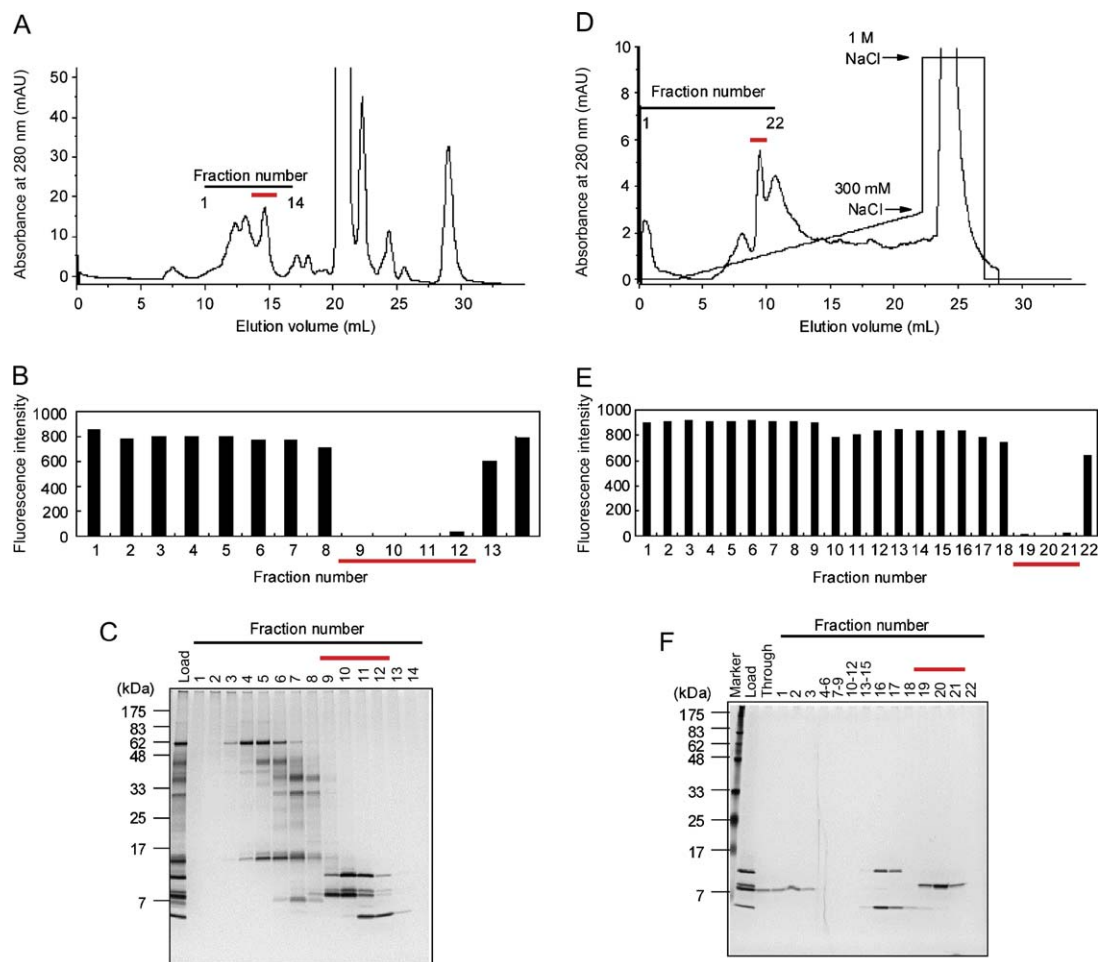


FIG 2. Purification of the inhibitor by means of size-exclusion (A-C) and anion-exchange (D-F) column chromatography. Fig 2, A and D, Elution profiles monitored by absorbance at 280 nm. Fig 2, B and E, Inhibitory activities of the separated fractions against proteolytic activity of Der f 1. Fig 2, C and F, SDS-PAGE. The fractions indicated with red lines were subjected to anion-exchange column chromatography (Fig 2, A-C) or used as the purified inhibitor (Fig 2, D-F).

Purification of the skin-derived inhibitor against the proteolytic activities of Der p 1 and Der f 1 from sweat

Human sweat concentrated with a YM10 membrane (Millipore, Bedford, Mass) with a nominal molecular limit of 10 kD was subjected to size-exclusion column chromatography (Superose 12 HR 10/30; Amersham Biosciences, Piscataway, NJ) by using PBS containing 0.01% sodium azide for elution and fractionation. All fractions were analyzed for their inhibitory activity against the proteolytic activity of Der f 1 with butyloxycarbonyl-Gln-Ala-Arg-MCA (Peptide Institute, Osaka, Japan)^{20,21} and were then subjected to SDS-PAGE, followed by silver staining (Silver Stain II kit; Wako, Osaka, Japan). The fractions with inhibitory activity were subsequently recovered, mixed, dialyzed against 25 mM Tris-HCl (pH 8.5) containing 0.005% sodium azide, and subjected to anion-exchange column chromatography (HiTrap QHP 1 mL; Amersham Biosciences). All fractions of NaCl gradient elution ranging from 0 to 300 mM and elution at 1 M NaCl were analyzed for inhibitory activity and then subjected to SDS-PAGE. The fractions with inhibitory activity were subsequently recovered, mixed, and stored at 4°C. They were then subjected to protein sequencing.²¹

Immunoblotting

Sweat from volunteers and the purified inhibitor were examined by means of SDS-PAGE under nonreducing conditions. After this, the samples were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, Mass). The membrane was blocked with BlockAce (Snow brand, Sapporo, Japan) at 4°C overnight and then incubated with mouse anti-human cystatin A mAb (Oxford Biotechnology, Oxfordshire, United Kingdom) at 1:1000 dilution for 90 minutes. After being washed 3 times with PBS containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Biosciences) at 1:1000 dilution for 90 minutes. Next, after being washed again, the membrane was developed with an ECL+ kit (Amersham Bioscience), and the resulting chemiluminescence was detected with a LAS-1000 (Fuji Film, Tokyo, Japan).

Measurement of cystatin A

The concentrations of cystatin A in the samples of sweat, purified samples, and cell-culture supernatants were determined by means of ELISA with a kit (KRKA, Novo Mesto, Slovenia).

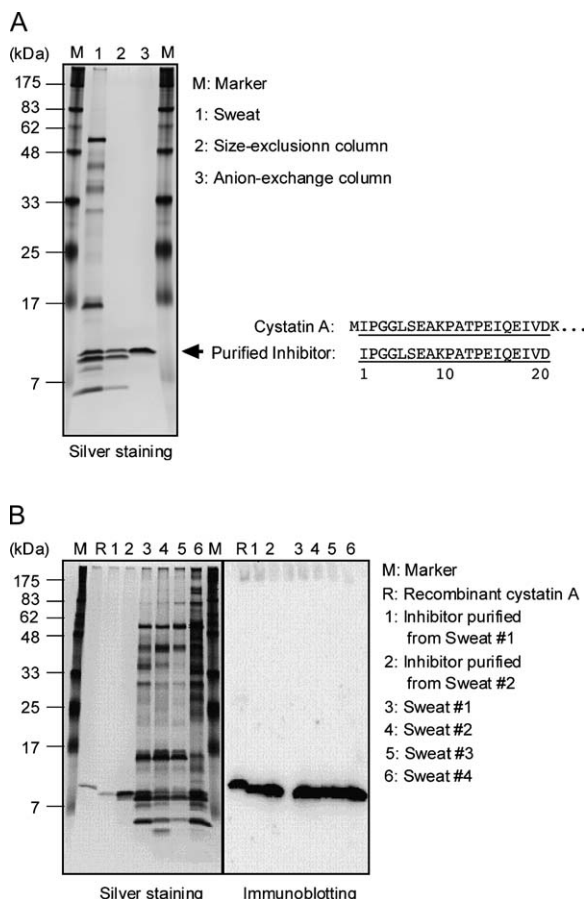


FIG 3. Identification of the inhibitor. **A**, The band for the purified inhibitor after size-exclusion and anion-exchange column chromatography analyzed by using N-terminal sequencing is indicated with an arrow. The N-terminal 20-amino-acid sequence of the inhibitor from sweat and the N-terminal sequence of cystatin A were compared. **B**, Immunoblotting with anti-human cystatin A mAb.

Removal of cystatin A from sweat

Protein G-sepharose beads (Amersham biosciences) were incubated with cystatin A-specific rabbit antibody (Biogenesis, Poole, United Kingdom) or nonspecific rabbit IgG (Chemicon, Temecula, Calif) overnight in a cold room with gentle rotation and then extensively washed with PBS. The antibody-coupled beads were incubated with PBS-diluted sweat for 4 hours to remove cystatin A from the sweat. The supernatant was recovered and incubated again with fresh antibody-coupled beads. The sweat after the second treatment with the beads was examined for inhibition of the proteolytic activity,²¹ with some modifications. The final concentration of the enzymes was 5 nM, and the fluorescence was measured at an excitation of 380 nm and emission of 460 nm on a fluorometer, SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, Calif).

Cell culture

Normal human keratinocytes from infant foreskins were purchased from Cascade Biologics (Portland, Ore) and cultured in serum-free HuMedia-KG2 keratinocyte growth medium (Kurabo Industries, Osaka, Japan) containing 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. Normal keratinocytes were used for experi-

ments at the third to fifth passages. Cells of the spontaneously immortalized human keratinocyte cell line HaCaT were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FCS and antibiotics. The keratinocytes were passaged by incubating the monolayer of the cells with prewarmed trypsin-EDTA solution for 5 to 10 minutes at room temperature. Trypsin-neutralizing solution (Cambrex, Walkersville, Md) was added to the cell suspension, and cells were formed into pellets by means of centrifugation at 1000 rpm for 5 minutes. After this, they were resuspended in culture medium, counted by means of trypan blue exclusion to determine the cell number and viability, and plated at 5×10^4 cells per well in 0.5-mL volumes in 24-well plates (Corning Incorporated, Corning, NY). The cultures were then incubated in 5% CO₂/95% air at 37°C.

Stimulation of keratinocytes

Keratinocytes grown to 80% confluency were washed, cultured in basal medium for 12 hours before stimulation, and then washed and incubated with Der p 1 or Der f 1 at 100 nM and an agonist peptide of the human protease-activated receptor-2 (PAR-2; SLIGKV-NH₂; Bachem, Bubendorf, Switzerland) at 500 μ M for normal keratinocytes and 100 μ M for HaCaT cells as a positive control.²³ All additives were prepared in basal medium prewarmed to 37°C. After preincubation with 5 mM L-cysteine at neutral pH for 10 minutes at 37°C, Der p 1 and Der f 1 were diluted in the basal media. In some experiments, the activated enzymes were diluted in basal medium containing recombinant human cystatin A (100 nM; R&D Systems, Minneapolis, Minn). The cells were incubated for 48 hours, and the supernatants were collected to determine IL-8 production and subjected to ELISA with a kit (DuoSet; R&D Systems). The data shown represent the means $\pm$ SEM of 3 wells. The significance of the differences in the mean responses was determined by using the 2-tailed unpaired *t* test.

RESULTS

Sweat inhibited the proteolytic activity of Der p 1 and Der f 1

The sweat from all 4 tested volunteers showed inhibitory activity against the cleavage of a synthetic substrate by Der p 1 and Der f 1 (Fig 1, A and B). On the other hand, the fractions passed through a 10-kD membrane showed no inhibitory effect (Fig 1, A, 10-kD fraction). These results suggested that the inhibitory effect of the sweat was not caused by changes of pH or ionic strength by the addition of sweat and that an inhibitor or inhibitors with a molecular weight of greater than 10 kD of membrane was present in the sweat. Moreover, their sweat showed inhibitory activities for cleavage of protein substrates by Der p 1 and Der f 1 for a collagen substrate (Fig 1, C), and the human low-affinity IgE receptor (CD23) and the α subunit of the human IL-2 receptor (CD25) on the cell surface (Fig 1, D and E). Sweat from one volunteer was used for the Azocoll assay (Fig 1, C). Sweat samples from 2 individuals were used for the cleavage assay of the cell-surface molecules, and similar results were obtained (Fig 1, D and E). The results shown in Fig 1 are representative of 2 or 3 independent experiments.

Purification of the inhibitor from sweat

Because we expected that the sweat contained various soluble skin-derived factors but smaller amounts of

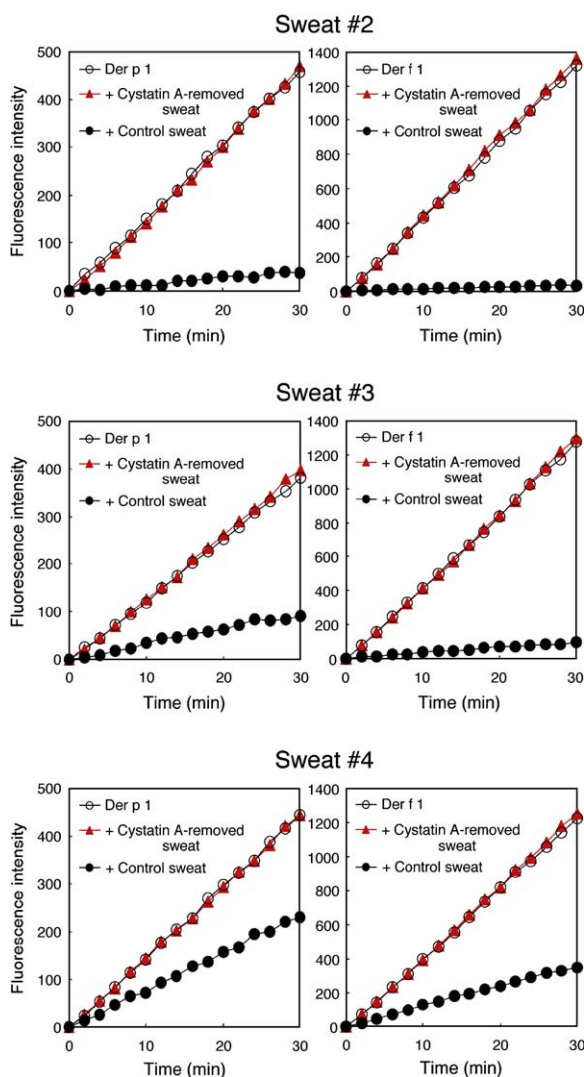


FIG 4. Removal of cystatin A eliminated the inhibitory activity of sweat against the proteolytic activities of Der p 1 and Der f 1. Diluted sweat treated with anti-cystatin A rabbit antibody-coupled beads (+ Cystatin A-removed sweat) or nonspecific rabbit IgG-coupled beads (+ Control sweat) was added to the allergens before reaction with a substrate, butyloxycarbonyl-Gln-Ala-Arg-MCA.

intracellular components than did the cell extracts, we chose sweat as the skin-derived material for isolation of the inhibitor. The inhibitor was purified by means of size-exclusion (Fig 2, A-C) and subsequent anion-exchange column chromatography (Fig 2, D-F). After this, it was detected as a single band by means of SDS-PAGE (Fig 2, F, lanes 19-21, and Fig 3, A, lane 3). Similar results were obtained by using sweat from 2 individuals for purification.

The inhibitor purified from sweat was cystatin A

The purified inhibitory fractions were subjected onto N-terminal amino acid sequencing, for which its N-terminal 20-amino-acid sequence was determined and

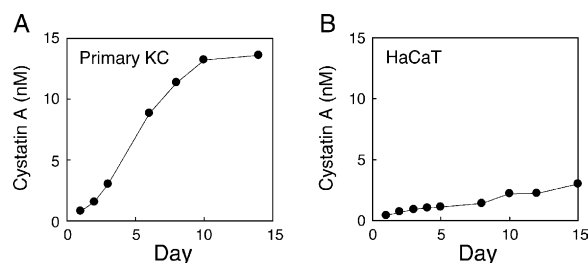


FIG 5. Secretion of cystatin A from keratinocytes into the culture supernatant. **A**, Primary cultured normal human keratinocytes (KC). **B**, HaCaT cells.

found to be identical to an internal sequence beginning from the residue just after the first methionine of human cystatin A (Fig 3, A), as well as epidermal-derived cystatin A reported by Takeda et al.²⁴ Similar results were obtained with sweat from 2 individuals for purification. Immunoblotting with anti-human cystatin A mAb also indicated that the purified inhibitor was cystatin A (Fig 3, B, lanes 1 and 2). The sweat from 4 volunteers contained cystatin A (Fig 3, B, lanes 3-6), but histidine-tagged recombinant cystatin A showed slightly less mobility (Fig 3, B, lane R).

Removal of cystatin A from sweat by anti-cystatin A antibody-coupled beads completely eliminated the inhibitory activity of the sweat from 3 volunteers tested, ruling out the potential nonspecific inhibitory effect of sweat and indicating that cystatin A is the exclusively dominant inhibitor (Fig 4).

The concentrations of cystatin A within the sweat determined by means of ELISA were 360, 113, 78, and 450 nM, and the recovered sweat volumes were 15, 150, 200, and 10 mL for sweat samples 1 through 4, respectively. Thus increase of the recovered volume resulted in the dilution of cystatin A, suggesting a possibility that this inhibitor was not so rich in original sweat secreted from sweat glands but was eluted mainly from the skin surface, the epidermis, or both by the running sweat.

Cultured keratinocytes secreted cystatin A into the medium

Primary cultured normal human keratinocytes secreted cystatin A into the medium dependent on the length of culturing (Fig 5, A), where less cystatin A was detected within the culture supernatant of HaCaT cells (Fig 5, B).

Der p 1 and Der f 1 stimulated IL-8 production by keratinocytes, which was blocked by cystatin A

To evaluate whether Der p 1 and Der f 1 stimulated the keratinocytes, IL-8 levels in the culture supernatant were measured. Normal keratinocytes secreted statistically significant levels of IL-8 when stimulated with Der p 1 or Der f 1 compared with medium alone, and the IL-8 levels were higher under culture conditions with a higher calcium concentration than with a lower one (Fig 6, A). The upregulation of IL-8 secretion by stimulated normal

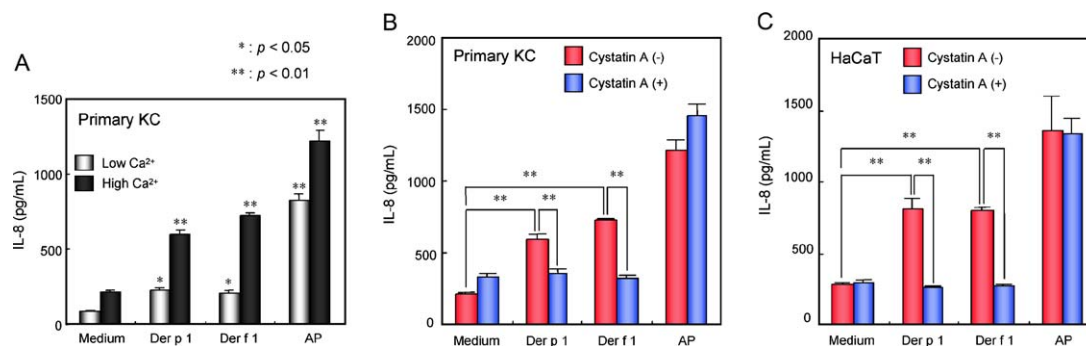


FIG 6. Der p 1 and Der f 1 stimulated IL-8 production by keratinocytes, and cystatin A blocked this. **A** and **B**, Primary cultured human normal keratinocytes (KC) were stimulated with Der p 1 and Der f 1 in original basal medium containing 0.15 mM CaCl₂ (panel A, Low Ca²⁺) or calcium-added basal medium containing 1.35 mM CaCl₂ (panel A, High Ca²⁺, and panel B). **C**, HaCaT cells. AP, Agonist peptide of PAR-2.

keratinocytes was blocked by the addition of cystatin A (Fig 6, B). We considered the blocking effect by cystatin A to be specific for the proteolytic activity of Der p 1 and Der f 1 because no blocking effect was observed for keratinocytes stimulated with the PAR-2 peptide agonist as a positive control (Fig 6, B, AP). Der p 1 and Der f 1 caused the upregulation of IL-8 secretion, and cystatin A blocked this for HaCaT cells, as well as normal keratinocytes (Fig 6, C). Cell detachment was not observed on stimulation with Der p 1 and Der f 1.

DISCUSSION

We found that human sweat inhibited the proteolytic activities of Der p 1 and Der f 1, and we used this as a skin-derived material for isolation of the protease inhibitor of Der p 1 and Der f 1, which was subsequently determined to be cystatin A. The stimulation of normal human keratinocytes and cells of the human keratinocyte cell line HaCaT with Der p 1 and Der f 1 upregulated IL-8 secretion, and the addition of cystatin A blocked this. These results indicate that cystatin A produced by keratinocytes is the dominant biochemical skin barrier, which eliminates the proteolytic activity of mite cysteine proteases and prevents them from stimulating keratinocytes.

At least 5 cysteine protease inhibitors, cystatin A (stefin A),^{24,25} cystatin M/E,²⁶ the prosequence of cathelicidin,²⁷ SCCA1, and SCCA2,^{7,28} exist on or are inducible in human skin. The first report on the inhibitory activity of sweat was by Yokozeki et al,²⁹ who isolated an inhibitor from sweat with minimal epidermal contamination by means of affinity purification with a papain-coupled column, although they did not determine the identity of the inhibitor. We used sweat possibly containing epidermal products as the starting material to purify the skin-derived inhibitor of the proteolytic activities of Der p 1 and Der f 1. Because we recovered fractions with inhibitory activity after size-exclusion or anion-exchange column chromatography without affinity purification using an enzyme-coupled column, our purification strategy indi-

cates the affinity between the inhibitor and enzyme and the amount of the inhibitor. The purified inhibitor was identified as cystatin A, and removal of cystatin A from sweat completely eliminated the inhibitory activity. Cystatin A shows more potent inhibitory activity against proteolytic activity of Der p 1 than other cystatins, including cystatin M/E,³⁰ and is strongly immunostained in the granular layer and moderately in the cornified and spinous layers in normal skin.²⁵ We also found that cystatin A was secreted into the culture supernatant of normal keratinocytes. Cystatin A purified from sweat was stable, even though it interacted with active Der p 1 or Der f 1 (unpublished observation). These results therefore indicate that cystatin A is the dominant skin-derived contributor in terms of affinity and the degree by which it can block the activity of the mite cysteine proteases.

Although human airway epithelial cells are stimulated by the cysteine protease activity of Der p 1 and the serine protease activity of Der p 3 and Der p 9 to upregulate their production of IL-8 and other proinflammatory cytokines through the activation of PAR-2,^{10,13,31} Mascia et al¹⁷ were unable to find such an *in vitro* effect with *D pteronyssinus* house dust mite extract in primary keratinocytes from healthy adults and adult patients with AD. However, we found that the proteolytic activities of Der p 1 and Der f 1 stimulate IL-8 production by primary keratinocytes from infants and HaCaT cells. This discrepancy between our results and those by Mascia et al might be from differences in conditions for culture and stimulation, keratinocytes used for experiments, and/or expression levels of the protease inhibitors. Although Asokanathan et al reported that the cysteine protease activity of Der p 1 stimulates airway epithelial cells through the activation of PAR-2,¹⁰ Kita and colleagues^{32,33} suggested that eosinophils respond to the cysteine proteases Der f 1 and papain through an as-yet-uncharacterized protease receptor. Whether the keratinocyte response to the cysteine protease activities of Der p 1 and Der f 1 observed in the present study occurs through PAR-2 or another target molecule or molecules should be addressed in future studies.

Sakata et al⁷ reported that SCCA2, which is upregulated in bronchial epithelial cells stimulated with IL-4 and IL-13, inhibits the cysteine protease activity of Der p 1 and Der f 1 and suggested that a system including activation and suppression of epithelial cell response against the proteolytic activities of Der p 1 and Der f 1 exists in the respiratory tract. We found that keratinocyte-derived cystatin A eliminated the proteolytic activities of Der p 1 and Der f 1 and prevented them from stimulating keratinocytes in the skin. Because cystatin A abundantly exists in human skin, it blocks the proteolytic activities of mite cysteine proteases under normal skin conditions. On the other hand, the barrier dysfunction is a major manifestation in AD,^{34,35} and Seguchi et al³⁶ reported that the cystatin A content is decreased in the lesional skin of patients with AD. Therefore we believe that keratinocytes could be stimulated by exposure to Der p 1 and Der f 1 if the skin barrier is disrupted and suggest the possible role of the cysteine protease activities of Der p 1 and Der f 1 in the pathogenesis of AD.

In summary, we found that a system including the activation and suppression of the keratinocyte response against Der p 1 and Der f 1 exists in the skin and that cystatin A produced by keratinocytes is the dominant biochemical skin barrier against the proteolytic activities of the mite cysteine proteases. These results suggest that keratinocytes could be stimulated by exposure to protease allergens if the skin barrier is disrupted, resulting in the loss of the inhibitor. This in turn implicates a relationship between the activities of Der p 1 and Der f 1 and the induction, propagation, or both of AD, for which the barrier dysfunction is a major manifestation.

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