

precisely determined thus far, whereas the worldwide prevalence of EPP has been reported to be 1:75,000–1:200,000 (Todd, 1994). Thus, a nationwide survey and genotyping of the large number of Japanese EPP families is recommended and would be required to elucidate the virtual penetrance and prevalence of EPP in Japan.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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The study protocol was approved by the Ethics Committee of Hirosaki University Hospital. Informed consent was obtained from the patients described in this paper. This study was conducted according to the Declaration of Helsinki Principles.

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Reduction of Skin Barrier Function by Proteolytic Activity of a Recombinant House Dust Mite Major Allergen Der f 1

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TO THE EDITOR

Exposure to house dust mite allergens is an important risk factor for the production of specific IgE and is associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (Platts-Mills and Chapman, 1987). House dust mite Group 1 allergens, Der f 1 from *Dermatophagoides farinae* and Der p 1 from *Dermatophagoides pteronyssinus*, are major allergens and belong to the papain-like cysteine protease family (Thomas *et al.*, 2002). 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, cleaving and/or interacting with cell-surface molecules and intrinsic protease inhibitors, and modulating the function of various cells (Comoy *et al.*, 1998; Shakib *et al.*, 1998; Gough *et al.*, 1999; Takai *et al.*, 2005a).

Although mite-derived proteolytic activities have been reported to disrupt the bronchial epithelial barrier (Herbert *et al.*, 1995; Wan *et al.*, 1999), whether they disrupt the skin barrier, which is considered a much more rigid barrier system, has not been investigated. Here, we test whether the proteolytic activity of Der f 1 causes a reduction in the barrier function of the skin in nude mice using a recombinant Der f 1 (rDer f 1) with full cysteine protease activity.

We demonstrated that rDer f 1 activated with L-cysteine reduced the barrier function of the skin in dose- and time-dependent manners (Figure 1) and that the reduction was dependent on its proteolytic activity (Figure 2). All animal studies have been approved by the Review Board of Juntendo University. By the use of nude mice, experimental procedures were simplified because of their hairless phenotype, and effects of

T cell-mediated acquired immunity on the barrier dysfunction could be ignored. The critical permeability function of the skin is mediated by the outermost layer of the epidermis, the stratum corneum (Strid and Strobel, 2005). The barrier function was evaluated based on two parameters, transepidermal water loss (TEWL) and the penetration by riboflavin of the stratum corneum. TEWL is a parameter for dryness of the skin, whereas riboflavin penetration is considered a parameter of the accessibility of the skin to environmental allergens and irritants. On Day 7, TEWL and riboflavin penetration were significantly increased by administration of 1 or 5 µg/site (Figure 1a) and 5 µg/site (Figure 1b) of activated rDer f 1, respectively, compared with the vehicle control. No increases were observed on Day 3 (Figure 1c and d). TEWL was greater at the patched site than water-treated site even in the vehicle control suggesting that the vehicle solution containing diluted

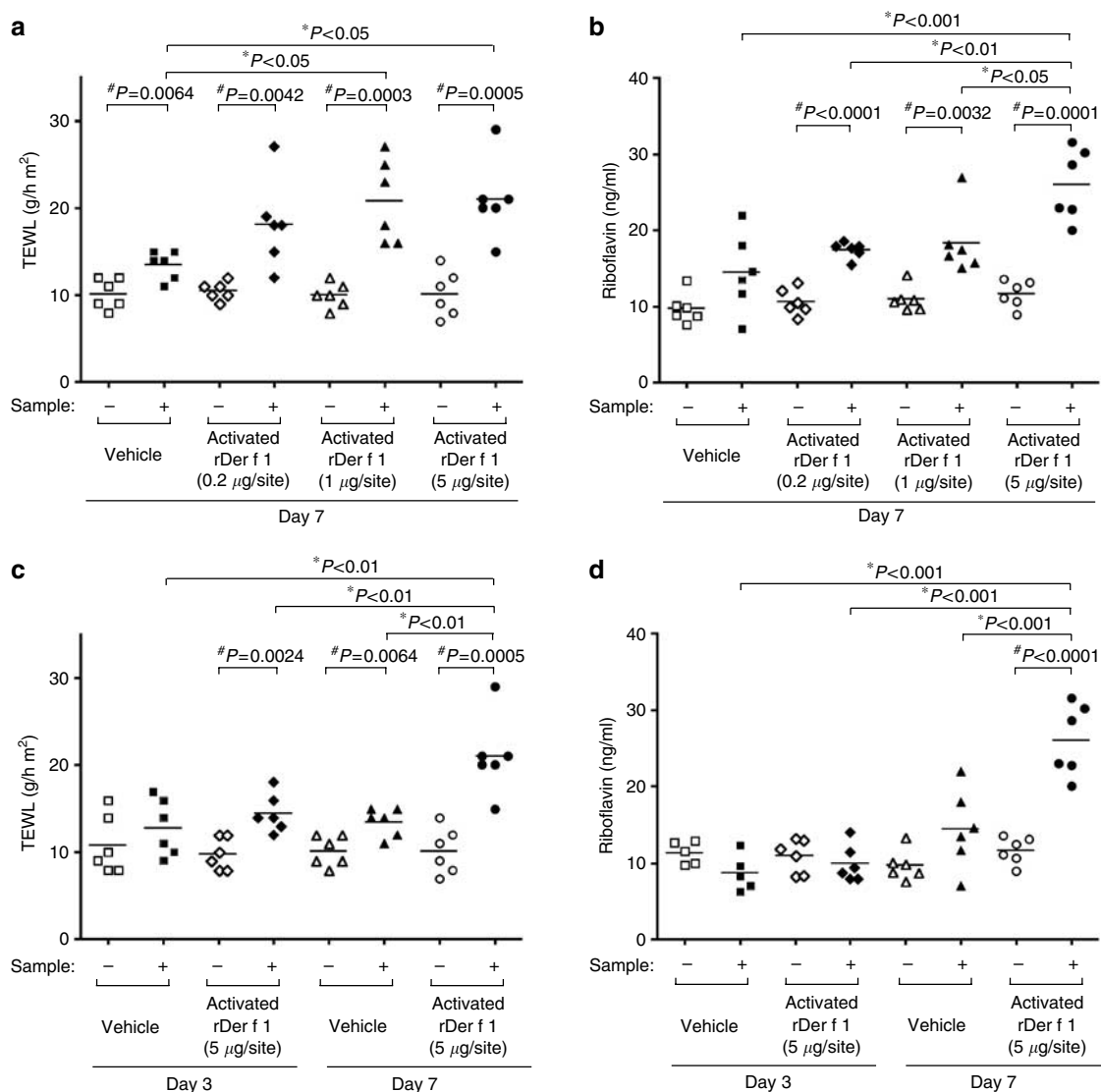


Figure 1. Dose- and time-dependent barrier dysfunction in the skin of nude mice at the sites where activated rDer f 1 was applied. Five-week-old female nude mice with the BALB/c background (Charles River Japan Inc., Yokohama, Japan) were maintained in a specific pathogen-free animal facility at Juntendo University. The glycosylated rDer f 1, designated Der f 1-WT, was expressed in yeast and prepared as described previously (Takai *et al.*, 2002, 2005b). The backs of mice anesthetized were lightly wiped with dry absorbent cotton once and cotton soaked with hexane twice. The use of hexane is to remove excess of scales. A rectangular piece of filter paper 1 cm × 2 cm (3MM Chr, Whatmann, Middlesex, UK) was placed on the back, and 100 μ l of solution containing rDer f 1 was perfused to the paper. The rDer f 1 was preincubated with 1.4 mM L-cysteine and diluted with phosphate-buffered saline and pure water. The final concentrations of rDer f 1 and L-cysteine were 50, 10, or 2 μ g/ml (5, 1, or 0.2 μ g/100 μ l/site) and 50 μ M, respectively, and the final dilution of phosphate-buffered saline was 13/100. The paper was wrapped with poly(vinylidene chloride) film cut to a size of 2 cm × 3 cm and then immobilized with an adhesive bandage (KINO SELF; Nitto Denko Co., Osaka, Japan). Solutions containing rDer f 1 activated with L-cysteine or the vehicle only was applied to mice (+). Another site towards the tail away from the sample-loaded site was loaded with pure water (–) and treated as like the sample-loaded site. The solutions for loading were administered a total of four times (Day 0, 2, 4, and 6) or twice (Day 0 and 2) onto the patch every other day. The (a and b) dose and (c and d) time influenced the levels of the barrier dysfunction. (a and c) TEWL. On the day after the last administration (Day 7 or 3), the bandage, Saran Wrap, and paper were removed and the back of each mouse was lightly wiped once with absorbent cotton soaked with hexane, and then TEWL was measured with a Mobile Tewameter (Courage + Khazaka electronic GmbH, Köln, Germany). (b and d) Riboflavin test. After the measurement of TEWL, the amount of riboflavin to penetrate the stratum corneum was measured as described previously (Okuda *et al.*, 2002) with some modifications as follows. Cotton soaked with 100 μ g/ml of riboflavin was placed on the site for 1 min and then removed, and the excess residual solution at the site was soaked up with wringed cotton. After drying for approximately 30 minutes, the stratum corneum was stripped with adhesive cellophane tape. The stripping was performed six times with the tape changed each time. The pieces of tape containing the stripped stratum corneum were dipped in 3 ml of a 1% sodium dodecyl sulfate solution overnight at 4°C protected from any light. The next day, the riboflavin was extracted from the tape by sonication. After vigorous mixing, the solutions were passed through a 0.2- μ m filter and moved to new tubes. The concentration of riboflavin was determined by measuring the fluorescence of riboflavin. The data shown represent the values for six or five mice. Bars indicate means. The Tukey's *post hoc* test followed by one-way analysis of variance was used to evaluate the significance of the differences among the sites treated with the samples (+) (*P<0.05). Unpaired Student's *t* test (two-tailed) was used to evaluate the significance of the differences between the sites treated with each sample (+) and pure water (–) (*P<0.05). A value of P<0.05 was regarded as statistically significant.

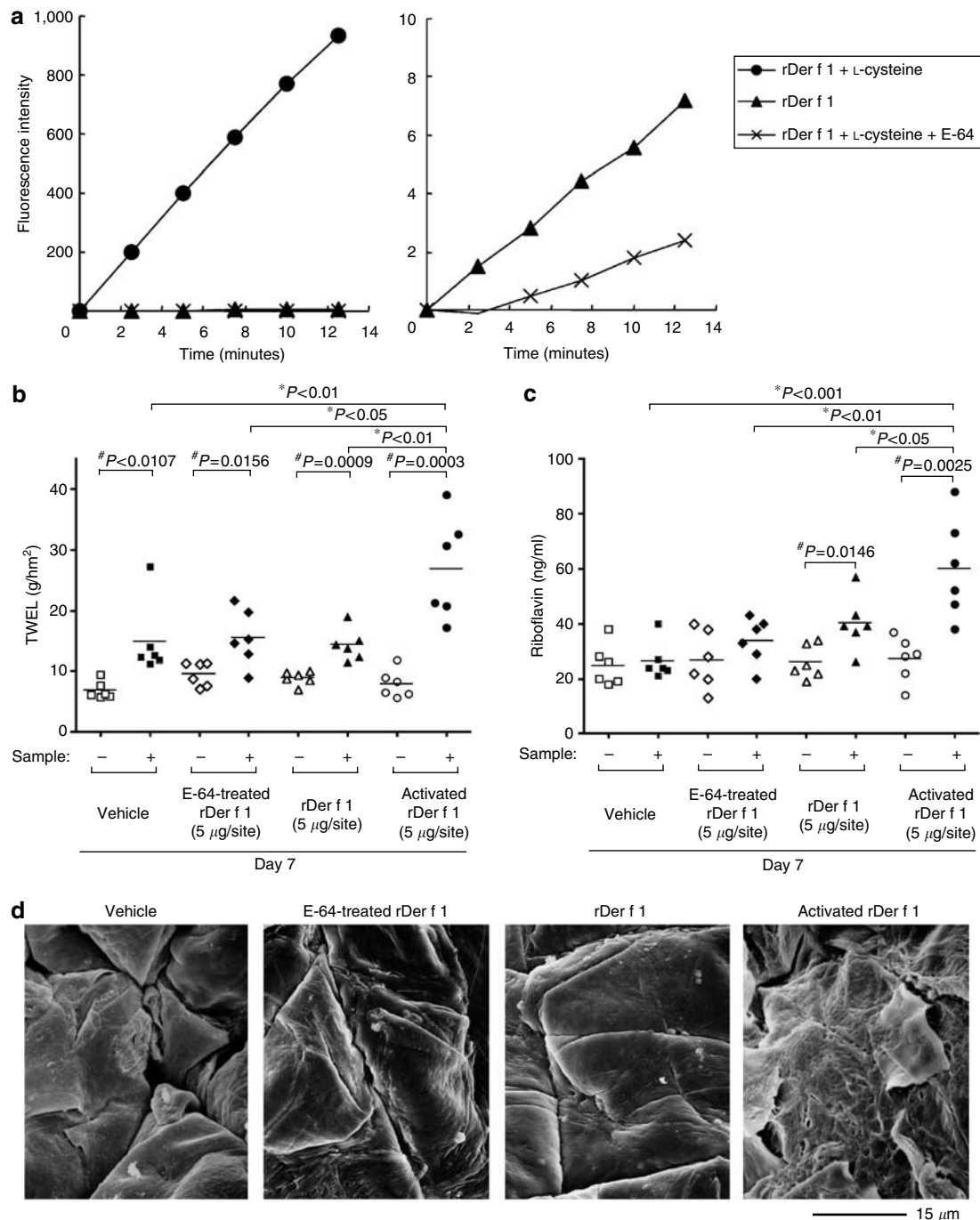


Figure 2. Barrier dysfunction and morphological changes in the skin of nude mice are dependent on the cysteine protease activity of rDer f 1. (a) Proteolytic activity of rDer f 1 before and after activation with L-cysteine, or after inactivation with an irreversible cysteine protease-specific inhibitor, E-64. The cysteine protease activity of rDer f 1 was measured as reported previously (Takai *et al.*, 2005c) except L-cysteine was used for the activation. Butyloxycarbonyl-Gln-Ala-Arg-MCA was used as the substrate. The scale of the horizontal axis in the right panel is 1/100 of that in the left panel. The activity of rDer f 1 in the absence of L-cysteine and in the presence of E-64 was, respectively, approximately 1/100 and 1/300 that of the fully activated rDer f 1. (b and c) Barrier dysfunction. To minimize the residual activity, E-64-treated rDer f 1 was prepared for administration to the mice by conducting the reaction with higher concentrations of rDer f 1 and E-64 and then dialyzing against phosphate-buffered saline. A solution containing rDer f 1 treated with L-cysteine (*Activated rDer f 1*), rDer f 1 not treated with L-cysteine (*rDer f 1*), a solution containing rDer f 1 treated with E-64 after its activation and then dialyzed against phosphate-buffered saline (*E-64-treated rDer f 1*) (5 μ g/100 μ l/site), or the vehicle only was applied to mice (+). Another site was loaded with pure water (-). The solutions for loading were administered four times onto the patch every other day (Day 0, 2, 4, and 6). On the day after the last administration (Day 7), measurement of TEWL (b) and riboflavin test (c) were performed. The data shown represent the values for six mice. The statistical analyses performed are as described in the legend of Figure 1. Data shown are representative of three independent patch experiments. (d) Scanning electron microscopy. Skin biopsies of the patched sites in mice not subjected to TEWL measurement and riboflavin test were analyzed.

phosphate-buffered saline and L-cysteine causes some disruption of the barrier as a background effect on Day 7 (Figure 1a and 2b). Although activated rDer f 1 significantly increased TEWL and riboflavin penetration and induced morphological changes at the sites where it was applied, rDer f 1 treated with an irreversible cysteine protease-specific inhibitor, E-64, did not (Figure 2b–d). Riboflavin penetration was greater at the sites exposed not only to activated rDer f 1 but also to unactivated rDer f 1 than at the water-treated sites (Figure 2c), indicating that the weak activity of the unactivated rDer f 1, which was not treated with L-cysteine, (Figure 2a) or the activity partially regenerated under the conditions on the patched skin was enough to cause minor barrier disruption.

An understanding of the mechanism of primary sensitization to allergens is important in elucidating the pathogenesis of allergic diseases and for possibly preventing their development. Mite allergens are an important factor in atopic dermatitis (Tan *et al.*, 1996; Friedmann, 1999; Kramer *et al.*, 2006), and Der f 1 and Der p 1 are present on the human skin (Yasueda *et al.*, 2003). Fecal pellets of *D. pteronyssinus* are known to contain Der p 1 at relatively high concentrations (10 mg/ml) (Tovey *et al.*, 1981) together with other mite serine proteases (Thomas *et al.*, 2002). Although it is difficult to relate the concentrations used in the present study to the amounts of mite proteases present on the human skin, we speculate that the amount of Der f 1 or Der p 1 at each point on the human skin, where mite fecal pellets directly attach, locally reaches to the amount applied in our experiments (four times administrations of $5 \mu\text{g}/100 \mu\text{l}/2 \text{ cm}^2/\text{site} = 20 \mu\text{g}/2 \text{ cm}^2$). The barrier dysfunction and morphological changes caused by the cysteine protease activity of rDer f 1 suggest that repeated exposure to mite bodies and fecal pellets could disrupt the local skin's barrier function. Areas of the skin where the barrier function is reduced are likely to provide a portal of entry for allergens and irritants that both initiate and perpetuate cutaneous inflammation. Recently, we have found that unglycosylated rDer f 1 and rDer p 1 activates cultured human keratinocytes, and a

skin-derived cysteine protease-specific inhibitor, cystatin A, can block the activation (Kato *et al.*, 2005) and that their proteolytic activity is crucial for IgE and IgG responses in mice (Takai *et al.*, unpublished observation; Kikuchi *et al.*, 2006). Taken together, the cysteine protease activity of the mite major group 1 allergens is now considered involved in the sensitization process in at least three ways, by reducing the barrier function of the skin and enabling allergens to penetrate, by stimulating keratinocytes, and by modulating immune responses. As no apparent epidermal proliferation and infiltration were induced on Day 7 in the present study using nude mice (unpublished observation), we consider that the barrier dysfunction was induced independently of inflammation. The process from the initial barrier disruption by mite proteases to inflammation with histological and immunological changes in atopic dermatitis is an important issue to be investigated in future studies.

As far as we know, this is the first demonstration of the barrier function of the skin being disrupted by the proteolytic activity of an allergen *in vivo*. Dry skin and an impaired barrier function are hallmarks of the pathogenesis of atopic dermatitis (Ogawa and Yoshiike, 1993; Leung *et al.*, 2004). Combination of barrier disturbance by a genetically determined barrier defect (Palmer *et al.*, 2006) and barrier disruption by some environmental allergens might increase the risk of sensitization to allergens toward IgE production and contribute to exacerbations of atopic dermatitis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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UV Increases the Nuclear Localization of Apurinic/Apyrimidinic Endonuclease/Redox Effector Factor-1 in Human Skin

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TO THE EDITOR

The apurinic-apyrimidinic endonuclease/redox effector factor-1 (APE/Ref-1) is a ubiquitous and bifunctional protein, which has both an apurinic-apyrimidinic endonuclease DNA repair activity and a nuclear redox activity (Tell et al., 2000a; Yang et al., 2005). Those two activities are split into two functionally independent domains of the protein: the N-terminus is principally devoted to the redox activity, whereas the C-terminus exerts enzymatic activity on abasic sites of DNA (Tell et al., 2005). Recently, it was reported that overexpression of APE/Ref-1 protects melanoma cells from cisplatin or from H₂O₂-induced apoptosis, whereas an increased rate of apoptosis is observed in the presence of APE/Ref-1 antisense. There are several previous reports detailing the subcellular localization of APE/Ref-1, which is translocated into the nuclei after exposure of human cells to oxidative stress (Tell et al., 2000b). Variable nuclear/cytoplasmic distributions of this

protein are observed in different human tissues (Freitas et al., 2003; Tanner et al., 2004). APE/Ref-1 has also been shown to control p53 activity through redox alteration. p53 is induced in cultured human skin fibroblasts after treatment with H₂O₂, an oxidant produced in cells during exposure to solar UV radiation (Vile, 1997). APE/Ref-1 is also linked to apoptosis, associated with thioredoxin. The thioredoxin reductase/thioredoxin system has been shown to translocate into the nucleus both in keratinocytes and in melanocytes (Hirota et al., 1999; Schallreuter et al., 2006). Therefore, the nuclear versus cytoplasmic distribution of APE/Ref-1 may be regulated by the environment.

We investigated the effects of UV on human skin of various pigmented phenotypes before and at different times after a single minimal erythral dose UV exposure. This study involved human subjects representing different racial/ethnic groups, and details of the protocols, subjects of the study,

and UV doses have been published previously (Tadokoro et al., 2003, 2005; Yamaguchi et al., 2006). This study was approved by the Food and Drug Administration (FDA) Research Involving Human Subjects Committee, and adhered to the Helsinki Guidelines; informed consent was obtained from each of the study subjects. A bank of FS lamps (National Biological Corp., Twinsburg, OH) was used as the source of UV radiation (60% UVA and 40% UVB). The relationship between UV exposure and apoptosis prompted us to investigate the expression of APE/Ref-1 (and its subcellular distribution) in human skin before and after UV exposure.

Immunohistochemical staining and quantitation of images was carried out as detailed previously (Tadokoro et al., 2003, 2005; Yamaguchi et al., 2006). Mouse anti-human APE/Ref-1 antibody (Affinity BioReagents, Golden, CO) was used at 4°C at a 1:100 dilution. Alexa and 4, 6-diamidino-2-phenylindole (DAPI) fluorescence was observed and analyzed using a Leica DMRB/DMLD laser microscope (Leica Microsystems,