

House Dust Mites Induce Production of Endothelin-1 and Matrix Metalloproteinase-9 in Keratinocytes via Proteinase-Activated Receptor-2 Activation

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Keywords

Atopic dermatitis · House dust mites · Keratinocytes · Endothelin-1 · Proteinase-activated receptor-2 · Matrix metalloproteinase-9

Abstract

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by skin barrier dysfunction and abnormal immune response. House dust mites (HDM) are a major source of allergens, some of which have cysteine and serine protease activities. Keratinocytes stimulated by HDM-derived proteases have been suggested to contribute to the pathogenesis of AD by producing various cytokines. However, whether keratinocytes contribute to the induction of pruritus in AD, especially by producing pruritus-related mediators upon stimulation with HDM-derived proteases, has not been fully elucidated. **Methods:** We examined whether the production of endothelin-1 (ET-1), matrix metalloproteinase (MMP)-2, and MMP-9 in keratinocytes can be induced by stimulation with *Dermatophagoides farinae* extracts, and if so, whether pretreatment with a protease inhibitor or proteinase-activated receptor-2 (PAR-2) antagonist affects the production of these mediators in keratinocytes. **Results:** Although MMP-2 levels were undetectable in

the culture supernatants, the production of ET-1 and MMP-9 was increased upon stimulation with HDM extracts in a concentration- and time-dependent manner and suppressed by pretreatment of HDM extracts with serine protease inhibitor, but not with cysteine protease inhibitor. Mite-derived serine proteases also induced ET-1 and MMP-9 production in a concentration- and time-dependent manner. Moreover, pretreatment with a PAR-2 antagonist inhibited the production of ET-1 and MMP-9 in keratinocytes. **Conclusion:** These results suggest that the activation of PAR-2 on keratinocytes by HDM-derived serine proteases induces the production of ET-1 and MMP-9, and may contribute to the induction of pruritus in AD.

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Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease affecting 10–20% of children and 1–3% of adults [1]. Its pathogenesis is contributed to interactions between susceptibility genes, the host's environment, skin barrier defects, and immunological factors [1]. Skin barrier defects in patients with AD are indicated to lead to dry skin and increased scratching, which cause

skin barrier breakdown and development of rash [2]. Keratinocytes are the largest cell population in the epidermis and play an important role in maintaining the epidermal barrier function. Moreover, it is reported that skin barrier breakdown results in exposure of keratinocytes to external stimuli and subsequent activation of keratinocytes, and that activated keratinocytes may contribute to the pathogenesis of AD by producing various cytokines and chemokines [3, 4].

Mites are now recognized as a major source of allergens in house dust [5]. Most patients with AD have high levels of specific immunoglobulin E (IgE) to house dust mite (HDM) allergens, and circulating mite-specific T cells are observed in the majority (70%) of mite-sensitive patients with AD [6, 7]. HDM allergens are divided into more than 10 specific groups on the basis of their biochemical function, sequence homology, and molecular weight [8, 9]. Among HDM-derived allergens, group 1 allergens and group 3, 6, and 9 allergens are shown to have cysteine and serine protease activities, respectively [9].

Proteinase-activated receptor-2 (PAR-2) is a G-protein-coupled receptor that is activated by specific proteolytic cleavage of the extracellular N-terminus. The newly formed N-terminus (tethered ligand) then interacts with the second extracellular loop, leading to conformational changes and consecutive signal transduction [10]. In lesional skin of patients with AD, PAR-2 is more homogeneously expressed by keratinocytes of all epidermal layers compared to normal skin [11, 12]. HDM-derived proteases are shown to activate PAR-2 on keratinocytes to produce cytokines (interleukin-8, granulocyte-macrophage colony-stimulating factor) [13]. Moreover, stimulation of keratinocytes with PAR-2 agonists has been reported to trigger release of thymic stromal lymphopoietin which acts directly on sensory neurons to induce robust itch-evoked scratching [14]. Purified trypsin, a serine protease, enhances the secretion of matrix metalloproteinases (MMP), which play an important role in neurite extension by degradation of extracellular matrix, in human keratinocytes via activation of PAR-2 [15]. These findings suggest that PAR-2 activation in keratinocytes by HDM-derived proteases can induce the production of endogenous pruritus-related mediators as well as inflammatory cytokines, which results in development of eczema with pruritus in patients with AD. However, the precise roles of keratinocytes in the induction of pruritus in patients with AD have not been fully understood.

Several mediators have been shown to play a crucial role in the induction of pruritus in AD [16]. Endothelin-1

(ET-1) is a 21-amino acid peptide which activates specific G protein-coupled ET_A and ET_B receptors [17]. ET-1 was originally characterized as a potent vasoconstrictor, but can also evoke itch sensations in humans [18]. Immunohistochemical studies have revealed the upregulation of ET-1 expression in the epidermis of patients with AD [19]. Moreover, its synthesis and release have been demonstrated in human keratinocytes in vitro [20]. These studies suggest that the increased expression of ET-1 in keratinocytes may contribute to the induction of itch sensation in patients with AD.

Several in vitro studies have demonstrated that the production of MMP-9, one member of the MMP family, is upregulated in keratinocytes upon stimulation with pruritogens and cytokines [21, 22]. Increased levels of MMP-9 are reported in the epidermis of involved skin in allergic contact dermatitis patients and skin wash samples from AD lesions [23, 24]. MMP-9 has been demonstrated to promote nerve growth factor (NGF)-induced neurite elongation [25]. It has been suggested that MMP-2 localized on the growth cone contributes to penetration of nerve fibers into the basement membrane in vitro [26]. The density of epidermal nerve fibers is higher in the skin of patients with AD than in healthy controls, which is suggested to be partly responsible for itch sensation in the periphery [27–29]. These observations suggest that MMP-2 and MMP-9 may be involved in the nerve fiber elongation observed in the lesional skin of AD patients, which leads to elevated itch sensation.

Therefore, to investigate the contribution of keratinocytes to the pathogenesis of AD, especially the induction of pruritus, we examined whether the production of ET-1, MMP-2, and MMP-9 in keratinocytes can be induced by stimulation with the extracts of *Dermatophagoides farinae* which is one of the most prevalent pyroglyphid mites found in house dust [8] and known to have cysteine and serine protease activities. Furthermore, we also examined the involvement of PAR-2 in the production of these mediators in keratinocytes.

Materials and Methods

Reagents

Lyophilized extracts of HDM, *D. farinae*, were purchased from COSMO BIO (Tokyo, Japan). [(2S,3S)-3-carboxyoxirane-2-carbonyl]-L-leucine (4-guanidinobutyl) amide hemihydrate (E-64; Peptide Institute, Osaka, Japan) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma-Aldrich, St. Louis, MO, USA) were used as a specific cysteine and serine protease inhibitor, respectively. Mite-derived serine proteases (MDSP) and selective PAR-2 antagonist (ENMD-1068) were purchased from

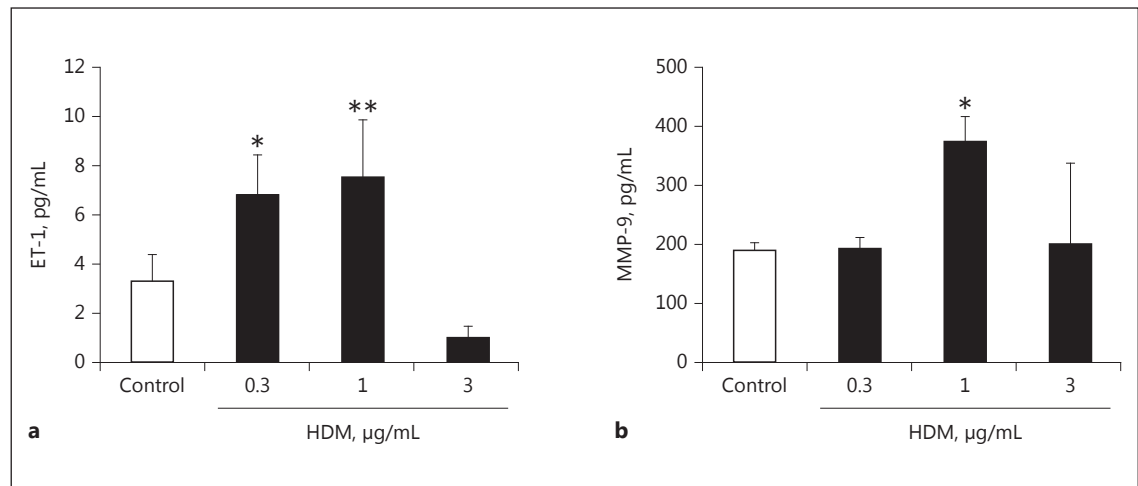


Fig. 1. HDM extracts induce the production of ET-1 and MMP-9 in human epidermal keratinocytes. Human epidermal keratinocytes were stimulated by HDM extracts for 48 h. ET-1 (**a**) and MMP-9 (**b**) levels in the culture supernatants were measured by ELISA. Each column represents the mean $\pm$ SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$ compared with vehicle-treated cells (control) (Dunnett multiple comparison test).

Indoor Biotechnologies (Charlottesville, VA, USA) and Enzo Life Sciences (Farmingdale, NY, USA), respectively.

Cell Culture

Primary human epidermal keratinocytes (Life Technologies, Carlsbad, CA, USA) were cultured in HuMedia-KG2 (Kurabo Industries, Osaka, Japan) supplemented with 10 μ g/mL insulin, 0.1 ng/mL human epidermal growth factor, 0.67 μ g/mL hydrocortisone hemisuccinate, 50 μ g/mL gentamycin, 50 ng/mL amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Keratinocytes were seeded in 24-well culture plates at 2×10^4 cells/well and grown to 80% confluency, and then cultured in basal medium (HuMedia-KB2; Kurabo Industries) supplemented with gentamicin and amphotericin B for 12–24 h and stimulated with HDM extract solutions (0.3, 1, or 3 μ g/mL) or MDSP solutions (0.3, 3, or 30 μ g/mL) for 1, 3, 6, 24, or 48 h. The lipopolysaccharide content in HDM and MDSP was undetectable by the ELISA kit (Cloud-Clone Corp., Houston, TX, USA). In some experiments, HDM extract solutions were pretreated with E-64 (50, 500, or 5,000 μ M) or AEBSF (5, 50, or 500 μ M) at 37°C for 15 min to inactivate HDM proteases before adding to the cultures. PAR-2 antagonist (0.1, 1, or 10 mM) was added to the cultures 6 h prior to the stimulation of cells by HDM extracts based on the previous report [30].

ELISA

After the incubation of cells with HDM extracts, cell culture supernatants were collected and stored at -80°C . The concentrations of ET-1, MMP-2, and MMP-9 in the supernatants were measured using Quantikine ELISA Kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance at 450 nm was determined by using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The detection limits of ET-1, MMP-2, and MMP-9 were 0.087, 33, and 156 pg/mL in these ELISA kits, respectively.

Cell Viability

Cell viability was evaluated by WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) assay (Cell Count Reagent SF; Nacalai Tesque, Kyoto, Japan), which is based on the bioreduction to a water-soluble colored formazan by dehydrogenases present in the viable cells. The amount of formazan product is directly proportional to the number of living cells. After collecting the culture supernatants, cells were replenished with the culture medium containing 10% WST-8 and incubated for 30 min at 37°C. Subsequently, the absorbance was measured at 450 nm using the microplate reader. The absorbance of the culture medium in the vacant wells was also measured as an arbitrary zero point.

Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation (SD) for 4 wells in each experiment. A significant difference between 2 groups was evaluated by the Student or Aspin-Welch t test following F -test. A Dunnett multiple comparison test was used for analysis of differences among 3 or more groups. A p value of less than 0.05 was considered statistically significant.

Results

ET-1 and MMP-9 Production Is Induced by Stimulation with HDM Extracts in Keratinocytes

To determine if HDM extracts can induce the production of endogenous pruritus-related mediators such as ET-1, MMP-2, and MMP-9 in keratinocytes, the levels of ET-1, MMP-2, and MMP-9 were measured in the culture supernatants collected at 48 h after stimulation with

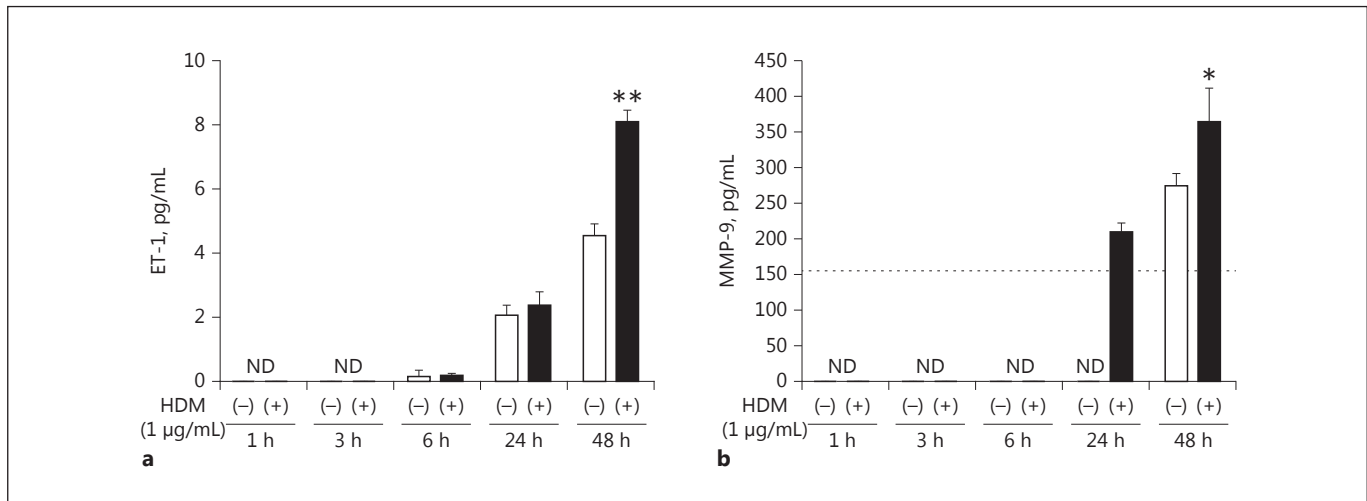


Fig. 2. ET-1 and MMP-9 production is time-dependently induced in human epidermal keratinocytes stimulated with HDM extracts. Human epidermal keratinocytes were stimulated by HDM extracts for 1, 3, 6, 24, or 48 h. ET-1 (a) and MMP-9 (b) levels in the culture supernatants were measured by ELISA. Each column represents

the mean $\pm$ SD ($n = 4$). ND, not detected. The dotted line indicates the lower limit of detection (156 pg/mL) in the MMP-9 ELISA kit. * $p < 0.05$, ** $p < 0.01$ compared with vehicle-treated cells (control) at each point (Student t test).

HDM extracts (0.3, 1 or 3 $\mu\text{g/mL}$). Although MMP-2 levels were undetectable at any stimulus concentration (data not shown), the production of ET-1 in keratinocytes was significantly increased upon stimulation with 0.3 and 1 $\mu\text{g/mL}$ HDM extracts in a concentration-dependent manner, but not affected with 3 $\mu\text{g/mL}$ HDM extracts (Fig. 1a). In contrast, the increase in MMP-9 levels was observed only after stimulation with 1 $\mu\text{g/mL}$ HDM extracts (Fig. 1b). Cell viability was not affected by 0.3 and 1 $\mu\text{g/mL}$ HDM extracts, but less than 80% of control at 3 $\mu\text{g/mL}$ HDM extracts (data not shown), indicating the decrease in ET-1 and MMP-9 levels at 3 $\mu\text{g/mL}$ HDM extracts was due to the cytotoxicity. Therefore, we determined to use 1 $\mu\text{g/mL}$ HDM extracts as the stimulus concentration in the following experiments.

Next, to investigate the time course of ET-1 and MMP-9 production in keratinocytes, the concentration of ET-1 and MMP-9 in the culture supernatants was measured after stimulation with 1 $\mu\text{g/mL}$ HDM extracts for 1, 3, 6, 24, or 48 h. ET-1 levels were increased in keratinocytes stimulated with HDM extracts depending on the culture time and resulted in significant increase after incubation for 48 h (Fig. 2a). Although MMP-9 levels were undetectable in the supernatants after incubation for 1, 3, and 6 h because of below detection limits, the elevation of MMP-9 levels in the supernatants was observed by HDM stimulation for 24 and 48 h in a time-dependent manner

(Fig. 2b). Cell viability was not affected by stimulation with HDM extracts at any time in culture (data not shown). Therefore, we chose 48 h as the culture time to investigate the mechanisms by which HDM extracts induce the production of ET-1 and MMP-9 in keratinocytes.

Proteolytic Activities of HDM Extracts Induce the Production of ET-1 and MMP-9 in Keratinocytes

To investigate whether proteolytic activities of HDM extracts are required for the production of ET-1 and MMP-9 in keratinocytes, the cells were stimulated with HDM extracts pretreated with E-64 (cysteine protease inhibitor) and AEBSF (serine protease inhibitor) for 48 h. E-64 at any concentration did not affect the production of ET-1 and MMP-9 (Fig. 3a, b). In contrast, AEBSF significantly inhibited ET-1 production in a concentration-dependent manner (Fig. 3c). The MMP-9 levels were also reduced by treatment with AEBSF at a concentration of 5 μM , and not detected when treating with 50 and 500 μM AEBSF because of falling below detection limits (Fig. 3d). Moreover, MDSP induced the production of ET-1 and MMP-9 in a concentration- and time-dependent manner (Fig. 4a–d), which is consistent with the above data. Cell viability was not affected by E-64 or AEBSF-treated HDM extracts, MDSP, and the addition of E-64 or AEBSF in the absence of HDM extracts (data not shown).

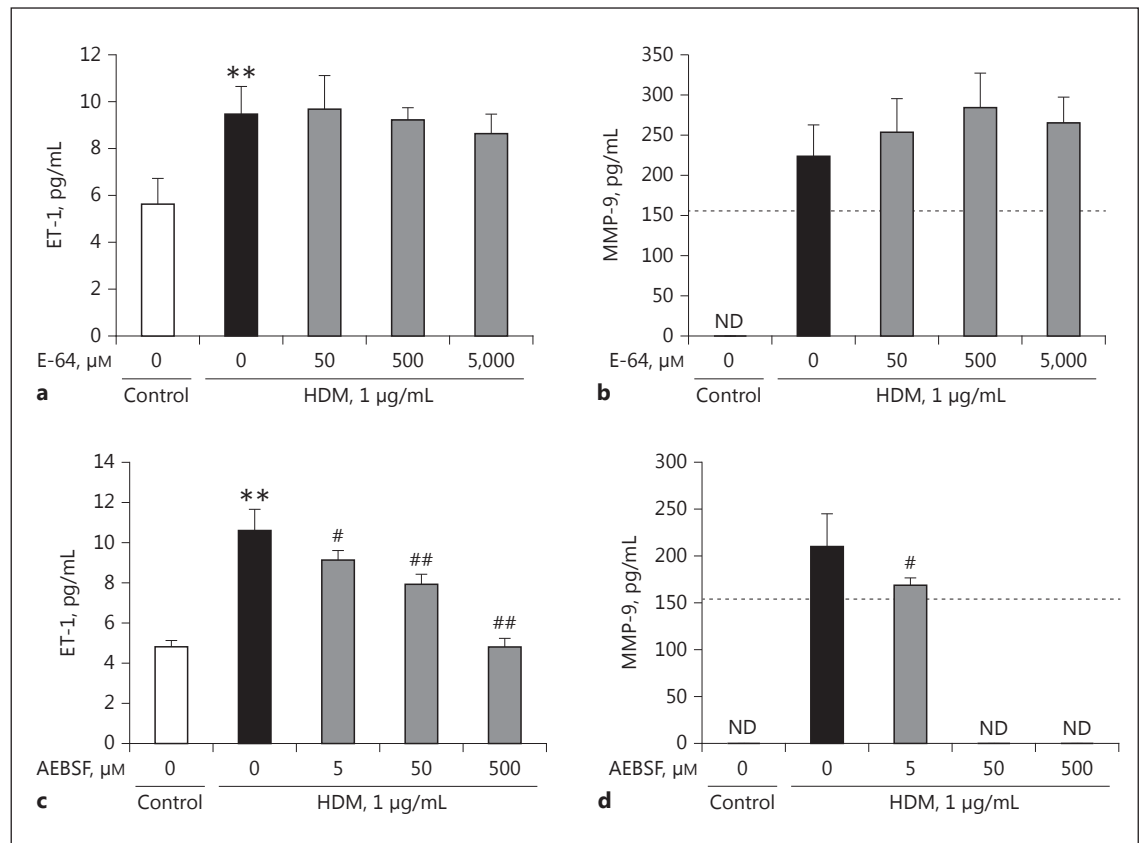


Fig. 3. The serine protease inhibitor, but not the cysteine protease inhibitor, suppresses ET-1 and MMP-9 production in human epidermal keratinocytes stimulated with HDM extracts. Human epidermal keratinocytes were stimulated by HDM extracts pretreated with E-64 (**a, b**) (cysteine protease inhibitor) or AEBSF (**c, d**) (serine protease inhibitor) for 48 h. ET-1 (**a, c**) and MMP-9 (**b, d**) levels in the culture supernatants were measured by ELISA. Each col-

umn represents the mean $\pm$ SD ($n = 4$). ND, not detected. The dotted line indicates the lower limit of detection (156 pg/mL) in the MMP-9 ELISA kit. ** $p < 0.01$ compared with vehicle-treated cells (control) (Student t test). # $p < 0.05$, ## $p < 0.01$ compared with HDM-stimulated cells (AEBSF, 0 μ M) (Dunnett multiple comparison test).

PAR-2 Activation Is Required to Induce the Production of ET-1 and MMP-9 in Keratinocytes upon Stimulation with HDM Extracts

Next, we investigated whether the activation of PAR-2 by HDM extracts is involved in ET-1 and MMP-9 production. Keratinocytes were incubated in the presence of ENMD-1068 (selective PAR-2 antagonist) for 6 h, and then stimulated with HDM extracts for 48 h. Pretreatment with ENMD-1068 significantly and concentration-dependently inhibited the production of ET-1 and MMP-9 in response to stimulation of HDM extracts (Fig. 5a, b). Cell viability was not affected by pretreatment with ENMD-1068 (data not shown).

Discussion

In the present study, we investigated whether HDM extracts induce the production of ET-1, MMP-2, and MMP-9 in keratinocytes via PAR-2-dependent mechanism. The production of ET-1 and MMP-9 in keratinocytes was increased upon stimulation with HDM extracts in a concentration- and time-dependent manner. The production of ET-1 and MMP-9 was not affected by treatment with the cysteine protease inhibitor, but was inhibited by the serine protease inhibitor. MDSP also induced ET-1 and MMP-9 production in a concentration- and time-dependent manner. Moreover, the production of ET-1 and MMP-9 was decreased in keratinocytes pretreated with the PAR-2 antagonist. Therefore, these find-

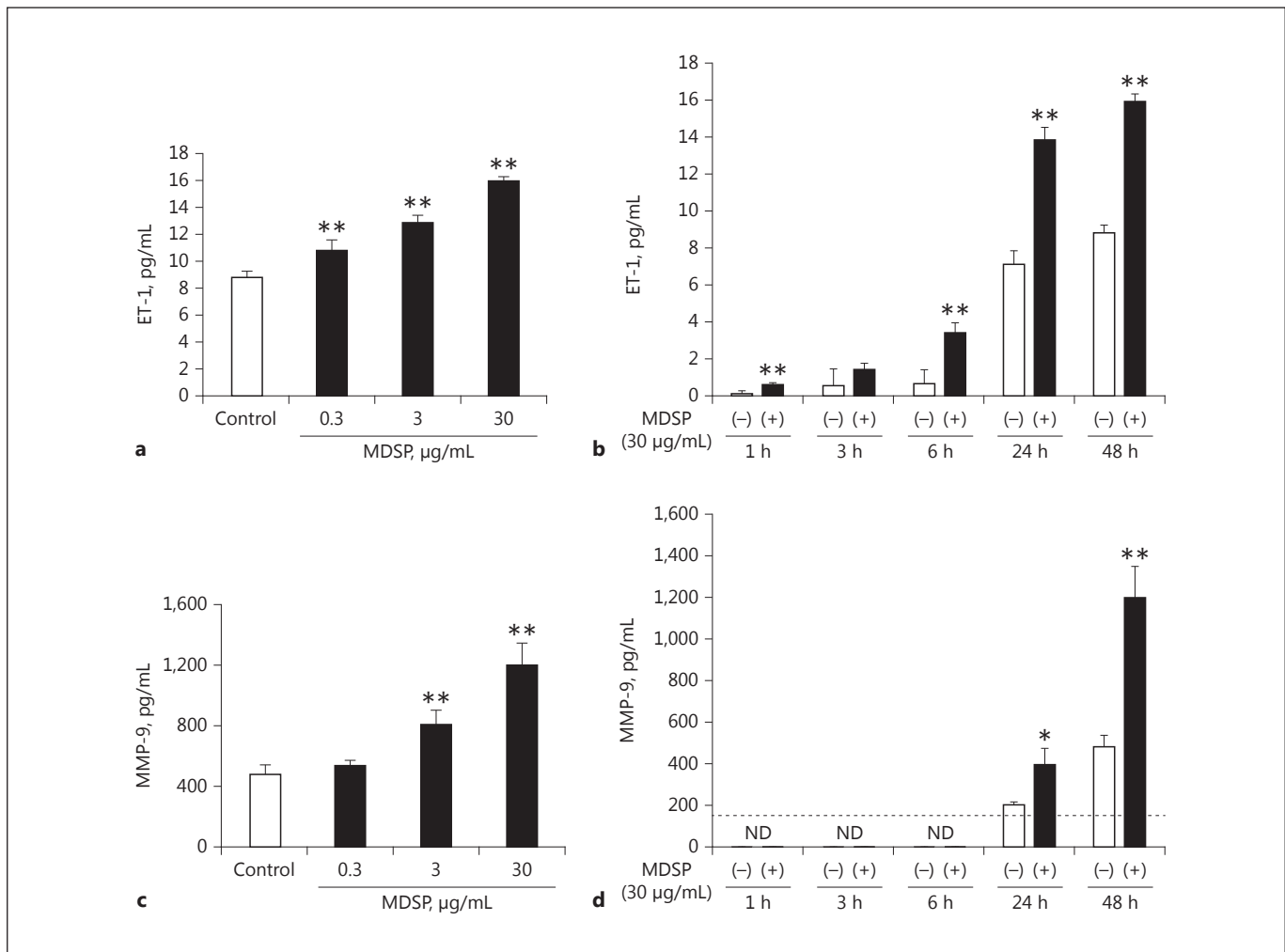


Fig. 4. Mite-derived serine proteases (MDSP) induce ET-1 and MMP-9 production in human epidermal keratinocytes in a concentration- and time-dependent manner. **a, c** Human epidermal keratinocytes were stimulated by MDSP for 48 h, and ET-1 (**a**) and MMP-9 (**c**) levels in the culture supernatants were measured by ELISA. ** $p < 0.01$ compared with vehicle-treated cells (control) (Dunnett multiple comparison test). **b, d** Human epidermal kera-

tinocytes were stimulated by MDSP for 1, 3, 6, 24, or 48 h, and ET-1 (**b**) and MMP-9 (**d**) levels in the culture supernatants were measured by ELISA. Each column represents the mean $\pm$ SD ($n = 4$). ND, not detected. The dotted line indicates the lower limit of detection (156 pg/mL) in the MMP-9 ELISA kit. * $p < 0.05$, ** $p < 0.01$ compared with vehicle-treated cells (control) at each point (Student or Aspin-Welch t test).

ings provide the first evidence that HDM-derived serine proteases may activate PAR-2 on keratinocytes to induce the production of ET-1 and MMP-9.

ET-1 has been shown to be a potent, partially histamine-independent pruritogen in humans [18]. Upregulation of ET-1 expression was reported in the epidermis of patients with AD compared with that of healthy controls [19]. Clinical studies have also demonstrated elevation of ET-1 levels in plasma that correlate with clinical severity, itch intensity, and serum IgE levels during AD exacerba-

tion [31]. On the other hand, MMP are a family of zinc-dependent endopeptidases capable of degrading extracellular matrix and basement membrane [32]. Degradation of extracellular matrix by MMP has been demonstrated to play an important role in neurite extension as well as cell migration and tissue remodeling. NGF-induced neurite sprouting was reported to be enhanced in the presence of MMP-9 relative to NGF treatment alone [25], suggesting the possibility that MMP-9 is involved in nerve growth by cooperating with NGF. Moreover, ele-

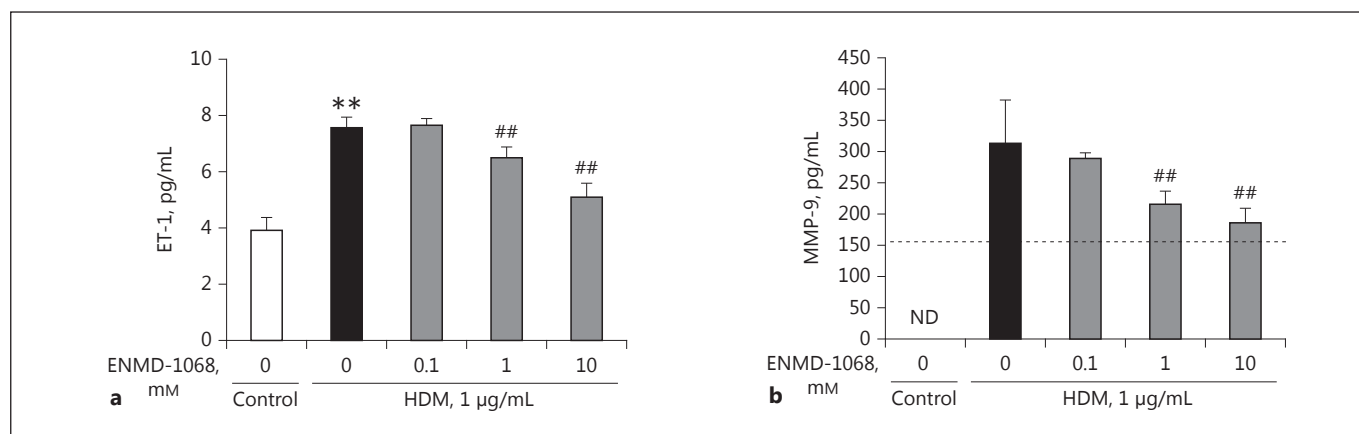


Fig. 5. The PAR-2 antagonist inhibits the production of ET-1 and MMP-9 in human epidermal keratinocytes stimulated with HDM extracts. Human epidermal keratinocytes were pretreated with ENMD-1068 (PAR-2 antagonist) for 6 h, and then stimulated by HDM extracts for 48 h. ET-1 (a) and MMP-9 (b) levels in the culture supernatants were measured by ELISA. Each column repre-

sents the mean $\pm$ SD ($n = 4$). ND, not detected. The dotted line indicates the lower limit of detection (156 pg/mL) in the MMP-9 ELISA kit. ** $p < 0.01$ compared with vehicle-treated cells (control) (Student t test); ## $p < 0.01$ compared with HDM-stimulated cells (ENMD-1068, 0 mM) (Dunnett multiple comparison test).

vated MMP-9 levels were reported in wash samples taken from the skin surface of patients with AD [23], and plasma MMP-9 levels were also found to be higher in patients with AD than in healthy controls [33]. Furthermore, the density of epidermal nerve fibers was found to be higher in the skin of patients with AD than in healthy controls, which is suggested to be partly responsible for itch sensation in the periphery [27–29]. These observations suggest that ET-1 and MMP-9 may contribute to the pathogenesis of AD, particularly the induction of pruritus. Therefore, our present study indicates the possibility that keratinocytes play a crucial role in the pathogenesis of AD by producing ET-1 and MMP-9 in response to stimulation of HDM extracts. In contrast, MMP-2 levels were undetectable in the culture supernatants in the present study. Although elevated levels of MMP-2 have been reported in involved skin of allergic contact dermatitis patients, MMP-2 was more weakly expressed than MMP-9 [24]. Moreover, there is no difference in the levels of MMP-2 in skin wash samples between AD patients and normal controls [23]. These findings assume that keratinocytes produce low levels of MMP-2 relative to MMP-9, which is consistent with the undetectable levels of MMP-2 in the present study.

HDM-derived allergens with proteolytic activities have also been reported to contribute to the pathogenesis of AD by producing cytokines through the activation of PAR-2 on keratinocytes [13]. HDM-derived allergens have been shown to have cysteine and serine protease ac-

tivities [9]. Therefore, we investigated the influence of pretreatment with a cysteine protease inhibitor, serine protease inhibitor, and PAR-2 antagonist in the production of ET-1 and MMP-9 in keratinocytes to clarify the involvement of HDM-derived protease activities in the production of endogenous pruritus-related mediators. The serine protease inhibitor, but not the cysteine protease inhibitor, markedly suppressed the production of ET-1 and MMP-9. Moreover, it was confirmed that MDSP induced the production of ET-1 and MMP-9. These results suggest that serine proteases derived from HDM may be involved in the production of ET-1 and MMP-9 in keratinocytes. Furthermore, the PAR-2 antagonist inhibited the production of ET-1 and MMP-9 in keratinocytes stimulated with HDM extracts, indicating the involvement of PAR-2 activation induced by HDM-derived serine proteases in the production of ET-1 and MMP-9 in keratinocytes. Additionally, as MDSP, group 3 and 9 allergens, have been reported to cleave the peptide corresponding to the N-terminus of PAR-2 at the activation site [34], it is assumed that the serine proteases in HDM extracts used in this present study also activate PAR-2 by cleaving the N-terminus peptide at the activation site.

ET-1 is also known to be an autocrine growth factor for keratinocytes [35, 36]. Therefore, the increase in ET-1 production in keratinocytes stimulated with HDM extracts in the present study implies the possibility that HDM-derived allergens may be also involved in enhanced

proliferation of keratinocytes in the skin of AD patients. Furthermore, the increase in MMP-9 production is reported to be induced by interleukin-8 produced by PAR-2 activation [13, 37], and it is assumed that cytokines produced by PAR-2 activation with HDM-derived serine proteases may also be involved in the increase of MMP-9 production in keratinocytes.

The expression of PAR-2 is also increased in mast cells in chronic skin inflammation [38], and PAR-2 activation is shown to increase histamine release from mast cells [39]. Furthermore, it is reported that histamine induces the expression of NGF in keratinocytes [40], which results in the promotion of nerve fiber sprouting [41]. Therefore, it is probable that the pruritus in patients with AD may be attributed to PAR-2 activation in both mast cells and keratinocytes induced by HDM-derived serine proteases.

Keratinocytes are the largest cell population in the epidermis and activated by various external stimulus such as HDM, and therefore keratinocytes are thought to play an important role in the pathogenesis of AD. In this present study, we first demonstrated that the production of ET-1 and MMP-9 from keratinocytes was induced upon stimulation with HDM extracts and inhibited by the serine protease inhibitor and PAR-2 antagonist. These findings suggest that the activation of PAR-2 on keratinocytes by HDM-derived serine proteases induces the production of ET-1 and MMP-9, and may contribute to the pathogenesis of AD.

Disclosure Statement

The authors state no conflict of interest.

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