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Involvement of Tryptase and Proteinase-Activated Receptor-2 in Spontaneous Itch-Associated Response in Mice With Atopy-like Dermatitis

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Abstract. This study investigated the involvement of tryptase and proteinase-activated receptor (PAR) subtypes in spontaneous scratching, an itch-associated behavior, in NC mice. This strain of mice showed chronic atopy-like dermatitis and severe spontaneous scratching, when kept a long time in a conventional environment. The trypsin-like serine proteinase inhibitor nafamostat mesilate (1 – 10 mg/kg) dose-dependently inhibited spontaneous scratching in mice with dermatitis. The activity of tryptase was increased in the lesional skin, which was inhibited by nafamostat at a dose inhibiting spontaneous scratching. Enzyme histochemistry revealed the marked increase of toluidine blue–stained cells, probably mast cells, with tryptase activity in the dermis of the lesional skin. Intravenous injection of anti-PAR_2 antibody suppressed spontaneous scratching of mice with dermatitis. Intradermal injection of the PAR_2-activating peptide SLIGRL-NH_2, but not $PAR_{1, 3, 4}$-activating peptides, elicited scratching at doses of 10 – 100 nmol/site in healthy mice. PAR_2-immunoreactivity was observed in the epidermal keratinocytes in healthy and dermatitis mice. These results suggest that PAR_2 and serine proteinase(s), mainly tryptase, are involved in the itch of chronic dermatitis.

Keywords: atopic dermatitis, itch, scratching, tryptase, proteinase-activated receptor

Introduction

Atopic dermatitis is a chronic inflammatory skin disease characterized by severe itch, a sensation that provokes a desire to scratch (1, 2). In atopic dermatitis patients, itch-elicited scratch aggravates dermatitis and increases itch more (3). Thus, it is important to control not only itch but also scratching in the treatment of atopic dermatitis. For the management of itch and scratch, we need to know the itch mechanisms of atopic dermatitis, which are still poorly understood. An H_1 histamine–receptor antagonist inhibits itch induced by mast cell degranulation in the skin of healthy subjects, but it is without effect in the lesional skin of patients with atopic dermatitis, suggesting that a mediator(s) other than histamine plays a key role in the itch of atopic dermatitis (4). Mast cell degranulation increases the cutaneous concentration of histamine, which is not apparently different between healthy skin and atopic eczema, but the concentration of tryptase is markedly increased in atopic eczema (4). Intradermal administration of a peptide that activates proteinase-activated receptor-2 (PAR_2) elicits pain and itch in healthy subjects, but it causes enhanced itch in atopic eczema (4). Since tryptase acts on PAR_2 (for review, see ref. 5), these findings raise the possibility that tryptase released from mast cells acts on PAR_2 in the skin to provoke itch in atopic dermatitis. However, the effects of blockade of tryptase activity and PAR_2 on the itch of atopic dermatitis have not been tested yet.

With regard to animal experiments, intradermal injection of tryptase potently elicits scratching, an itch-related response, in healthy mice, which is inhibited by an anti-PAR_2 antibody and a PAR_2 antagonist (6). Scratching induced by mast cell degranulation is also inhibited by the anti-PAR_2 antibody and the PAR_2 antagonist (6).

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Intradermal injection of PAR_2-activating peptide elicits scratching in mice (7, 8). Activating peptides of PAR_1 and PAR_4, but not PAR_3, also elicit scratching, but scratching induced by PAR_1- and PAR_4-activating, but not PAR_2-activating, peptides is inhibited by an H_1 histamine–receptor antagonist, suggesting that PAR_1- and PAR_4-associated scratching involves histamine release from mast cells (8). Thus, it is interesting to investigate whether tryptase and PAR subtypes are involved in the itching of chronic dermatitis.

When NC mice are kept a long period under a conventional environment, they develop spontaneously severe skin lesion, hyperplasia, increase in serum IgE level, and mast cells in the skin and severe scratching, an itch-associated behavior (9, 10); these features are similar to those of patients with atopic dermatitis (1, 2). In the present experiments, therefore, we investigated whether tryptase and PAR_2 are involved in itch-associated behavior in NC mice with chronic dermatitis.

Materials and Methods

Animals

We used male NC/jic mice (16 – 25-week-old) that were bred in a specific pathogen-free environment at the Division of Animal Resources and Development, Life Science Research Center, University of Toyama. They were kept under controlled temperature (23 ± 1°C), humidity (60 ± 5%), and light (light on 8:00 – 20:00 h). Food and water were freely available. Most mice were transferred to conventional environment at 4 – 5 weeks of age, and then they were kept together with mice with chronic dermatitis for 2 weeks for mite infection (10). These mice showed severe dermatitis after one or two months. Some mice were kept in the specific pathogen-free environment throughout the experiments.

Agents

Nafamostat mesilate (Torii Pharmaceutical Co., Ltd., Tokyo) was dissolved in 5% glucose solution and was injected intravenously 5 min before behavioral observation and tissue isolation. TFLLR-NH_2, SLIGRL-NH_2, SFNGGP-NH_2, and AYPGKF-NH_2, synthesized by JBL, were dissolved in saline and injected intradermally in a volume of 50 μl into the rostral back. Anti-PAR_2 antibody (SAM-11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse non-specific IgG were dissolved in phosphate-buffered saline and injected intravenously 1 h before behavioral observation.

Behavioral observation

Before the behavioral observation, the animals were placed individually in an acrylic cage composed of four cells (13 × 9 × 40 cm) for at least 1 h for acclimation, and then their behaviors were videotaped for 1 or 2 h with personnel kept out of the observation room. For the observation of evoked scratching, the animals were given an intradermal injection after a 1-h acclimation period and immediately put back into the same cells, and then the scratching toward the injected site by the hind paw was counted (11). For observation of spontaneous scratching, the animals were continuously kept in the same cells and scratching toward any regions of the body by the hind paws was counted (10). Since mice rapidly scratch several times for about 1 s, a series of these movements was counted as one bout of scratching (11).

Determination of tryptase activity

Activity of tryptase was measured as described previously (6). Briefly, the skin was isolated from the rostral back and homogenized in 10 mM Tris (tris (hydroxymethyl) aminomethane), pH 6.1, containing 2 M NaCl. After centrifugation, 1 μl of the supernatant (5 μg protein/μl) was added to 49 μl of sample buffer (0.06 M Tris-HCl, pH 7.8, containing 0.4% dimethyl sufoxide and 30 μg/ml heparin). The cocktail was reacted with 50 μl of substrate solution, 480 μg/ml N-*p*-Tosyl-Gly-Pro-Arg-*p*-nitroanilide (Sigma, St. Louis, MO, USA), in the sample buffer at 37°C for 1 h. *p*-Nitroanilide released was determined colorimetrically at 420 nm.

Western blotting

The skin was isolated from the rostral back and served for the determination of PAR_2 protein by Western blotting. Protein extraction, electrophoresis, and membrane transfer were done as described (12). The transferred membrane was reacted with anti-PAR_2 monoclonal antibody (SAM-11, 1:500; Santa Cruz Biotechnology) and anti-β-actin monoclonal antibody (1:5,000, Sigma). After washing with Tris-buffered saline, pH 7.5, containing 0.1% Tween 20, the membrane was reacted with horseradish peroxidase–conjugated anti-mouse IgG antibody (1:5,000; Amersham Bioscience, Piscataway, NJ, USA) for 90 min at room temperature. After washing with the Tris-buffered saline containing 0.1% Tween 20, it was reacted with chemiluminescence reagents (Amersham Bioscience) and signals were detected using X-ray film. The density of the band was analyzed with the NIH image program. The data was normalized to β-actin.

Immunohistochemistry

The animals were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The skin of the rostral back was removed, postfixed for 4 h in the same fixative, and kept overnight

in PBS containing 30% sucrose at 4°C. Frozen tissue was cut on a cryostat at a 30-μm thickness. The sections were washed with PBS containing 0.2% Tween 20 and incubated with rabbit anti-PAR_2 antibody (H-99, 1:200; Santa Cruz Biotechnology) for 24 h at 4°C. After washing with PBS containing 0.2% Tween 20, the sections were incubated with Alexa Flour 488–conjugated anti-rabbit antibody (1:1000; Molecular Probes, Grand Island, NY, USA) for 2 h at room temperature. The fluorescence signal was observed by using a confocal microscope (Bio-Rad, Hercules, CA, USA).

Enzyme histochemistry

Skin isolation, fixation, and sectioning were done as described above. Tryptase activity–positive cells in the section were detected according to the method described by Wolters et al. (13). Briefly, the sections (10 μm) were washed with 0.1 M phosphate buffer (pH 6.5) and incubated with the phosphate buffer containing 0.25 μg /μl Z-Gly-Pro-Arg-4-methoxy-2-naphtylamide (Bachem, Bubendorf, Switzerland) and 3 mM Fast Blue B at 37°C for 1 h. After the observation of enzyme activity, the section was counter-stained with 0.1% toluidine blue for the observation of mast cells.

Data analyses

Data were presented as means and S.E.M. Statistical significance was analyzed using Dunnett's multiple comparisons or Student's *t*-test, except for the time course, which was analyzed with the one way repeated measures analysis of variance followed by *post hoc* Dunnett's test. $P<0.05$ was considered significant.

Results

Effect of nafamostat on spontaneous scratching of mice with dermatitis

NC mice with chronic dermatitis showed marked spontaneous scratching (Fig. 1). The trypsin-like serine proteinase inhibitor nafamostat mesilate (1 – 10 mg/kg) produced a dose-dependent inhibition of the spontaneous scratching with significant effect at a dose of 10 mg/kg (Fig. 1). Nafamostat mesilate at a dose of 10 mg/kg did not affect locomotor activity (data not shown).

Effect of anti-PAR_2 antibody on spontaneous scratching of mice with dermatitis

To investigate the role of PAR_2 in spontaneous itch of dermatitis, an anti-PAR_2 antibody, which had been shown to inhibit tryptase-induced scratching (6), was injected into mice with chronic dermatitis. An intravenous injection of the anti-PAR_2 antibody (0.3 mg/kg) significantly suppressed spontaneous scratching during the interval from 2 – 3 h after injection (Fig. 2). An injection of non-specific IgG did not significantly affect the spontaneous scratching (Fig. 2).

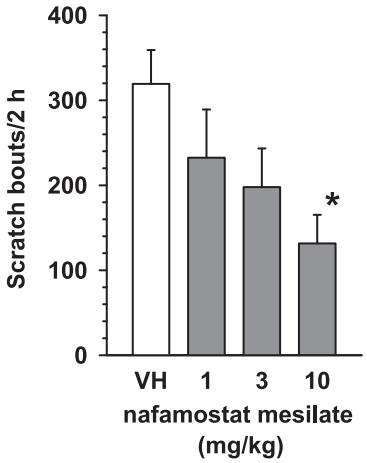

Fig. 1. Effects of nafamostat mesilate on spontaneous scratching of mice with dermatitis. Nafamostat mesilate or vehicle (VH, 5% glucose) was injected intravenously 5 min before the start of observation. Values each represent the mean and S.E.M. for seven to eight animals. *$P<0.05$, when compared with VH (Dunnett's multiple comparisons).

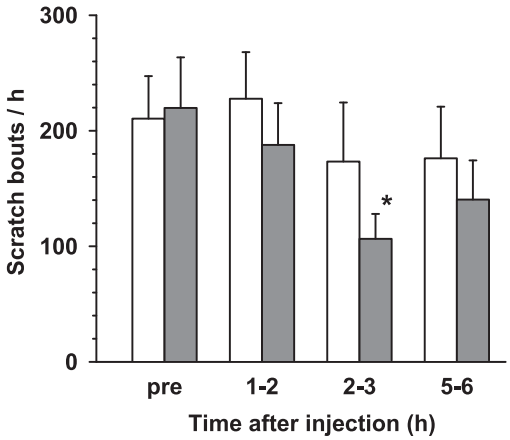

Fig. 2. Effect of anti-PAR_2 antibody on spontaneous scratching in mice with dermatitis. The mice were given an intravenous injection of non-specific IgG (0.3 mg/kg, open columns) and or anti-PAR_2 antibody (0.3 mg/kg, closed columns). Values each represent the mean and S.E.M. for eight animals. *$P<0.05$, when compared with pre-injection (Dunnett's multiple comparisons).

Scratching behavior after injection of PAR subtype–activating peptides in healthy mice

When injected intradermally at a dose of 100 nmol /site, the PAR_2-activating peptide SLIGRL-NH_2 elicited scratching; the effect was marked during the initial 20-min period (Fig. 3A). SLIGRL-NH_2 (10 – 100 nmol/site)

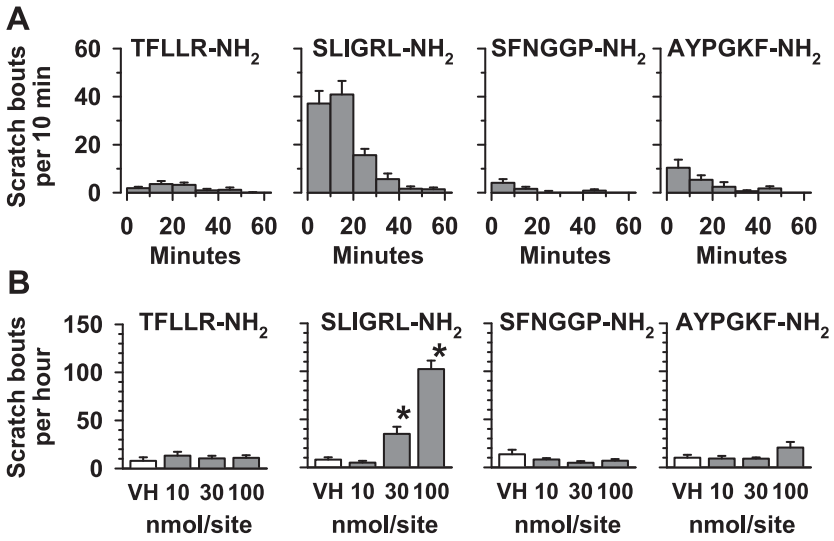

Fig. 3. Scratching after intradermal injection of PAR subtype–activating peptides in healthy mice. A: Time course of the effects of the peptides (100 nmol/site). B: Dose–response effects for the peptides. Scratch bouts were counted for 1 h after the intradermal injection of TFLLR-NH_2, SLIGRL-NH_2, SFNGGP-NH_2, AYPGKF-NH_2 (PAR_{1-4}-activating peptides, respectively), or vehicle (VH: saline). Values each represent the mean and S.E.M. for 7 – 8 animals. *P<0.05, when compared with VH (Dunnett's multiple comparisons).

produced a dose-dependent increase in scratching (Fig. 3B). The other PAR-activating peptides, TFLLR-NH_2 (PAR_1), SFNGGP-NH_2 (PAR_3), and AYPGKF-NH_2 (PAR_4), were without effects at the same doses (Fig. 3).

Effect of nafamostat on the increased activity of tryptase in the lesional skin

The activity of tryptase was significantly increased in the lesional skin of mice with chronic dermatitis as compared with the skin of healthy mice (Fig. 4A). Nafamostat mesilate (10 mg/kg) significantly reduced the increased tryptase activity in the lesional skin down to the level of the healthy skin (Fig. 4B).

Distribution of tryptase in the lesional skin

The distribution of tryptase in the skin was examined by enzyme histochemistry using tryptase-specific substrate peptide. There were few cells with active tryptase in the skin of healthy mouse (Fig. 5A). In contrast, there were many cells positive for active tryptase in the lesional skin (Fig. 5B). The positive cells were mainly distributed in the dermis and under the basal layer, and most cells with active tryptase were stained with toluidine blue (Fig. 5: B and C). Tryptase activity in the cell was markedly lower in the superficial dermis than in the deep dermis (Fig. 5B).

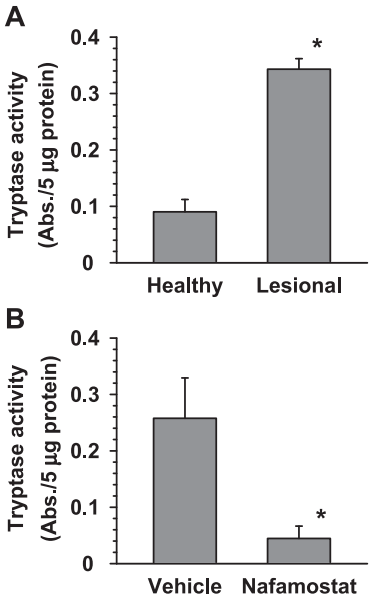

Fig. 4. Effect of nafamostat on the increased activity of tryptase in the lesional skin. A: Tryptase activity in the skin of the rostral back of mice with healthy skin or dermatitis. B: Effect of nafamostat on the increased activity of tryptase in the lesional skin. Nafamostat mesilate (10 mg/kg) or vehicle (VH: 5% glucose) was injected intravenously. The lesional skin was removed 5 min after the injection. The height of the columns represents absorbance per 5 μg protein. Values each represent the mean and S.E.M. for four (A) or six (B) animals. *P<0.05 (Student's t-test).

Distribution of PAR_2 in the skin

In the healthy skin, PAR_2-like immunoreactivity was present throughout the epidermis except the basal layer, suggesting that keratinocytes in the epidermis except the stratum basal layer express PAR_2 (Fig. 6A). In the lesional skin, epidermal hyperplasia was marked and PAR_2-like immunoreactivity was present throughout the expanded epidermis except for the basal layer (Fig. 6B). The intensity of the immunoreactivity was not apparently different between the healthy and lesional skin (Fig. 6: A and B). Western blot analysis confirmed the similarity in the expression level of PAR_2 in the keratinocytes between the healthy and lesional skin (Fig. 6C). PAR_2-like immunoreactivity was slightly observed in the

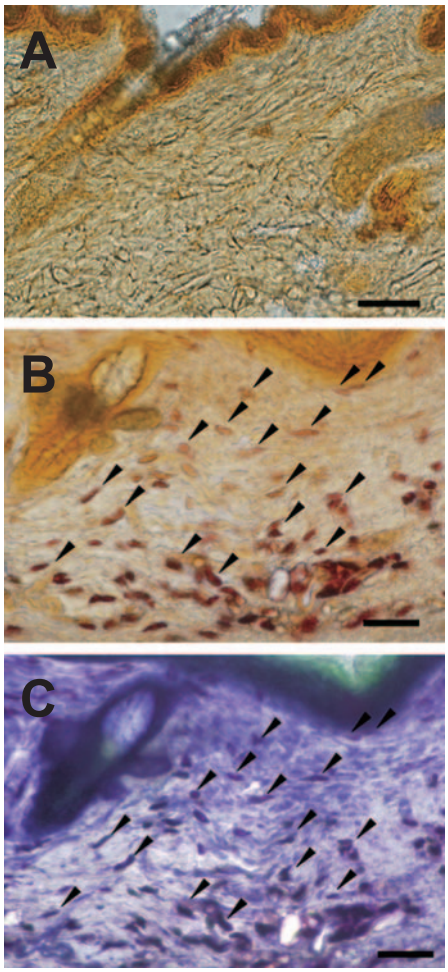

Fig. 5. Distribution of cells with active tryptase in the skin. Tryptase activity in the healthy (A) and lesional (B) skin was stained by enzyme histochemistry using Z-Gly-Pro-Arg-4-methoxy-2-naphtylamide as a substrate. C: Counter staining of the section of B with toluidine blue. Arrowheads point to cells positive for both tryptase activity and toluidine blue–staining. Scale bar = 50 μm.

hair follicles, but there were no clear nerve fiber–like structures positive for PAR_2-like immunoreactivity in the dermis of the healthy and lesional skin (Fig. 6: A and B).

Discussion

An intradermal injection of SLIGRL-NH_2, a PAR_2-activating peptide, elicited scratching in NC mice, but activating peptides of the other PAR subtypes were without effects, suggesting that only PAR_2 is involved in cutaneous itch. In contrast to NC mice, ICR mice show slight scratching in response to activating peptides of PAR_1 and PAR_4, but the responses to PAR_1- and PAR_4-, but not PAR_2-, activating peptides, were suppressed by the H_1 histamine–receptor antagonist, suggesting that

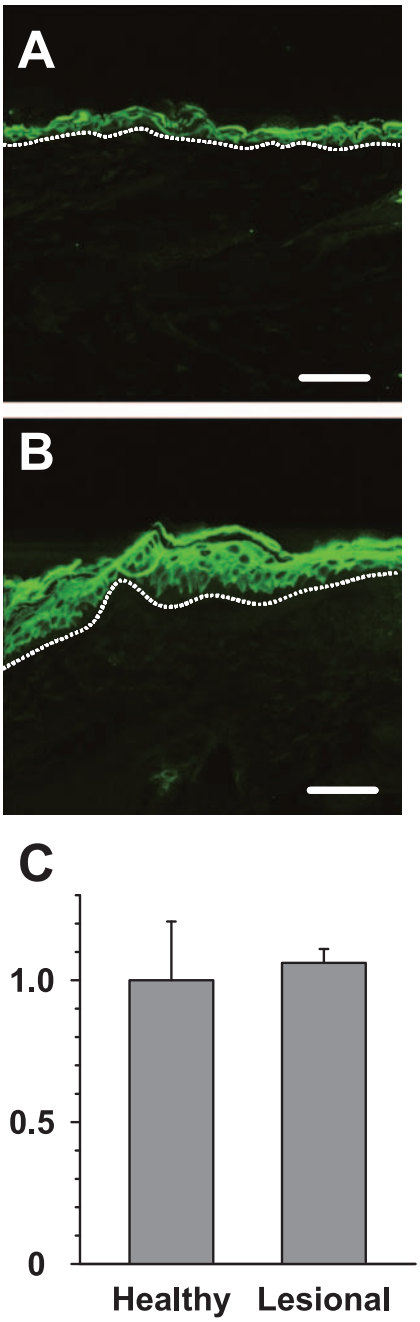

Fig. 6. Localization of PAR_2 in the mouse skin. PAR_2 was immunostained in the healthy (A) and lesional (B) skin. Dotted lines show the boundary between the dermis and epidermis. Scale bar = 50 μm. C: Expression level of PAR_2 in the skin. PAR_2 was determined by Western blotting and normalized to the expression level of β-actin. Values are each the mean and S.E.M. for six animals.

the PAR_1 and PAR_4 responses are mediated by histamine released from mast cells (8). An intradermal injection of histamine elicits scratching in ICR mice, but not in NC mice (10, 14, 15). Therefore, the activation of PAR_1 and PAR_4 could cause the degranulation of mast cells to release histamine, but even so, they might not elicit

scratching in NC mice. Since intradermal injections of PAR_1- and PAR_4-activating peptides may degranulate mast cells (8), these peptides were predicted to release mast cell tryptase to elicit scratching in healthy NC mice. However, they did not elicit scratching in healthy NC mice at a dose (100 nmol/site) eliciting scratching in ICR mice (8). In this regard, there were few cells with active tryptase and low tryptase activity in the skin of healthy NC mice. In addition, scratching elicited by PAR_1- and PAR_4-activating peptides is slight in ICR mice (8). Therefore, a presumable explanation for this discrepancy is that the amount of tryptase released by stimulation of PAR_1 or PAR_4 was not enough to elicit scratching.

The spontaneous scratching of mice with chronic dermatitis was suppressed by systemic administration of anti-PAR_2 antibody, which had been shown to inhibit scratching induced by tryptase (6). This result suggests that PAR_2 activation is involved in itching of chronic dermatitis. PAR_2 was markedly expressed in the epidermal keratinocytes of the healthy and lesional skin. The result is similar to PAR_2 localization in the skin of healthy humans and patients with atopic dermatitis (16). Thus, it is suggested that the epidermis keratinocytes are major target cells of PAR_2-acting proteinases. Keratinocytes release several itch mediators and enhancers such as leukotriene B_4, thromboxane A_2, and nitric oxide (17 – 19). Therefore, PAR_2-acting proteinases may induce itch through an action on the keratinocytes. The expression level of PAR_2 in the keratinocyte was not increased in mice with chronic dermatitis, but increase in the number of keratinocytes expressing PAR_2 in the epidermis might increase the response to PAR_2 stimulation. This may be a cause of the increased itch response to PAR_2-activating peptide in patients with atopic dermatitis (4). It remains unknown whether the stimulation of PAR_2 on the keratinocytes increases the production and release of any itch mediator(s) in the lesional skin. We will elucidate these issues for the understanding of pruritus of atopic dermatitis in the near future.

PAR_2 was present in the epidermis except for the basal layer in the mice. The results are similar to the human skin, in which intense immunoreactivity for PAR_2 was observed in the upper epidermis, especially in the granular layer and the immunoreactivity in the basal and spinous layers were low (16). Keratinocytes and melanocytes are major cells in the basal layer of the epidermis, in which about 10% of the cells are stem cells and about 50% are amplifying cells. Therefore, the result that PAR_2 immunoreactivity was hardly detected in the basal layer of the epidermis suggests that melanocytes, stem cells, and amplifying cells express little or no PAR_2. In this context, human melanocytes were claimed not to express PAR_2 (16).

PAR_2 mRNA has been detected in neurons in the rat dorsal root ganglion (20) and PAR_2-positive nerve fibers have been observed in the skin of patients with atopic dermatitis (4), suggesting the presence of PAR_2-expressing primary afferents in the skin. In contrast, we observed no nerve fiber-like structures positive for PAR_2 in the healthy and lesional skin of the mouse. The present results do not support the presence of PAR_2-expressing nerve fibers in the skin of NC mice, but they do not deny the idea that PAR_2-expressing nerve fibers are involved in the itch of patients with atopic dermatitis (4).

The activity of tryptase and the number of mast cells with active tryptase were markedly increased in the dermis of lesional skin of mice with chronic dermatitis. These results are similar to a report on human skin showing that tryptase-containing mast cells are markedly increased in the dermis of patients with atopic dermatitis (16). Enzyme histochemistry can not reveal the degranulation of mast cells, but tryptase activity was markedly lower in the cells in the superficial dermis than in the deep dermis of the lesional skin, suggesting that mast cells in the superficial dermis had released tryptase. The spontaneous scratching of mice with chronic dermatitis was inhibited by nafamostat at a dose that reversed the increased activity of tryptase in the skin. The same dose inhibited scratching induced by intradermal injection of tryptase, without effects on scratching induced by histamine and serotonin (6). Nafamostat also inhibits scratching induced by compound 48/80, which degranulates mast cells, and this inhibition is almost abolished by deficiency in mast cells, suggesting that the action on mast cells is responsible for the inhibitory action of nafamostat (6). This agent inhibits serine proteinases, including trypsin, C1 esterase, and thrombin; and it most potently inhibits tryptase with a K_i value of 0.1 nM (21). Scratching induced by intradermal injections of tryptase and compound 48/80 is suppressed by anti-PAR_2 antibody, suggesting that tryptase released from mast cells acts on PAR_2 to induce itch (6). With these findings taken into account, the present results suggest that the inhibition of mast cell tryptase plays an important role in nafamostat's suppression of itching in chronic dermatitis.

The human stratum corneum epidermis contains members of the kallikrein family, which may contribute to degradation of intercellular adhesion molecules, resulting in the desquamation of corneocytes (22). Among kallikrein members, kallikrein 8, a trypsin-like serine proteinase, is increased in the striatum corneum and serum, suggesting the increased secretion (23). Transgenic mice expressing kallikrein 7, a chymotrypsin-

like serine proteinase, in the epidermis, in addition to striatum corneum, gradually develop chronic dermatitis and spontaneous scratching (24). However, kallikreins 7 and 8 do not activate PAR_2 (25) and nafamostat is 1,000 times or more potent against tryptase than plasma kallikrein (21, 26). Therefore, these kallikreins may not be molecules primarily involved in the suppressive action of nafamostat on the itching of chronic dermatitis.

Chronic topical application of tacrolimus alleviates pruritus of patients with atopic dermatitis (27). The chronic anti-pruritic effects may involve anti-immune and anti-inflammatory actions (for review, see ref. 28), but it may also have acute anti-pruritic action. For example, acute topical application of tacrolimus inhibits scratching induced by PAR_2-activating peptide, but not histamine (29). In this context, scratching induced by SLIGRL-NH_2, a PAR_2-activating peptide, is not inhibited by an H_1 histamine–receptor antagonist (7, 8), and distinct dorsal horn neurons may receive signals of cutaneous stimulation with PAR_2-activating peptide and histamine (30). Taking these findings into account, the present results suggest that PAR_2 and tryptase are interesting target molecules for the development of novel drugs effective against anti-histamine-resistant pruritus, like that in atopic dermatitis.

Acknowledgments

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