Involvement of Serine Protease and Proteinase-Activated Receptor 2 in Dermatophyte-Associated Itch in Mice

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ABSTRACT
We investigated the involvement of serine protease and protease-activated receptor 2 (PAR2) in dermatophyte-induced itch in mice. An intradermal injection of an extract of the dermatophyte Arthroderma vanbreuseghemii (ADV) induced hind-paw scratching, an itch-related behavior. ADV extract-induced scratching was inhibited by the opioid receptor antagonists naloxone and naltrexone, the serine protease inhibitor nafamostat mesylate, and the PAR2 receptor antagonist FSLLRY-NH2. ADV extract-induced scratching was not inhibited by the H1 histamine receptor antagonist terfenadine or by mast cell deficiency. Heat pretreatment of the ADV extract markedly reduced the scratch-inducing and serine protease activities. Proteolytic cleavage within the extracellular N terminus of the PAR2 receptor exposes a sequence that serves as a tethered ligand for the receptor. The ADV extract as well as tryptase and trypsin cleaved a synthetic N-terminal peptide of the PAR2 receptor. The present results suggest that serine protease secreted by dermatophytes causes itching through activation of the PAR2 receptors, which may be a causal mechanism of dermatophytosis itch.

Introduction
Superficial cutaneous fungal infections, especially tinea, are very common in dermatological foot diseases, and cause skin conditions, such as scales, keratosis, erosion, and itching; itching is reported by approximately 50% of patients with tinea pedis (Cohen et al., 2002; Djeridane et al., 2006). Fungi proteinase has long been known to be pruritogenic in humans (Arthur and Shelley, 1955). Dermatophyte infection leads to immediate and delayed-type hypersensitivities (Woodfolk, 2005), which can cause pruritus. However, the details of the underlying mechanisms of dermatophytosis remain poorly understood. Dermatophytes secrete a variety of enzymes, such as proteinases, lipases, elastases, collagenases, phosphatases, and esterases, which are important factors during the infection process (Peres et al., 2010).

Therefore, we first aimed to determine whether dermatophyte products, especially proteases, cause acute itching.

Proteases have long been known to cause itching in humans; moreover, endopeptidases rather than exopeptidases cause itching (Arthur and Shelley, 1955). Among the endopeptidases (proteinases), serine proteases may cause itching via proteinase-activated receptor (PAR), a family member of the G-protein-coupled receptors. The activation of PAR is initiated by the cleavage of the N terminus of the PAR receptor to generate a new tethered ligand for the receptor. The ADV extract as well as tryptase and trypsin cleaved a synthetic N-terminal peptide of the PAR2 receptor. The present results suggest that serine protease secreted by dermatophytes causes itching through activation of the PAR2 receptors, which may be a causal mechanism of dermatophytosis itch.

ABBREVIATIONS: PAR, proteinase-activated receptor; ADV, A. vanbreuseghemii; FK888, N2-[(4R)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-N-methyl-N-phenylmethyl-3-(2-naphthyl)-L-alaninamide.
ulation are involved in the itching induced by dermatophyte products.

Materials and Methods

Animals. Male ICR mice (5–9 weeks old or neonatal) were used, with the exception of one series of experiments in which male mast cell-deficient mice (WBB6F1 W/W) and the normal littermates [WBB6F1 (+/+)] were used at 8 weeks of age. All mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a room under controlled temperature (21–23°C), humidity (45–65%), and light (lights on from 7:00 AM to 7:00 PM). Food and water were made freely available. Procedures in the animal experiments were approved by the Committee for Animal Experiments at University of Toyama and were conducted in accordance with the guidelines of the Japanese Pharmacological Society.

Materials. Naloxone hydrochloride and naltrexone hydrochloride (both from Sigma-Aldrich, St. Louis, MO) were dissolved in physiological saline and injected subcutaneously 15 min before dermatophyte extract injection. Terfenadine (Sigma-Aldrich) was dissolved in 0.5% sodium carboxymethyl cellulose (Wako Pure Chemical Industries, Osaka, Japan) and administered orally 30 min before Arthroderma vanbreuseghemii (ADV) extract injection. Nafamostat mesylate (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in 0.5% sodium carboxymethyl cellulose (Wako Pure Chemical Industries) and injected intravenously 5 min before ADV extract injection. The peptides FSLLRY-NH₂, SLIGRL-NH₂, LRGILS-NH₂, GRNNSKGRSLIGRLET-NH₂, and GRNNSKGIILGRLET-NH₂ were synthesized and identified using the peptide synthesizer PSSM-8 (Shimazu Co., Kyoto, Japan) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer Autoflex T1 (Bruker Daltonics, Billerica, MA), respectively. The resultant pellet was suspended in physiological saline (Ohtsuka Pharmaceutical Co., Ltd., Tokushima, Japan) and injected intradermally together with ADV extract. N-p-Tosyl-Gly-Pro-Arg p-nitroanilide was dissolved in 50 mM Tris-HCl, pH 8.0, in a concentration of 0.5 mg/ml. A 0.02-ml volume of ADV extract or heat-treated ADV extract was added to 0.18 ml of the substrate solution, and the mixture was incubated at 37°C for 1 h. The amount of p-nitroanilide released was colorimetrically determined at 420 nm.

Activity of PAR₂ Cleavage. Ten micrograms of GRNNSKGRSLIGRLET-NH₂ (an N-terminal peptide of PAR₂ containing a tethered ligand sequence SLIGRL) and its analog GRNNSKGIILGRLET-NH₂ (two amino acids, Arg-Ser, of trypsin-like serine protease-cleaved site were replaced by Ile-Ile) were reacted with ADV extract (10-µg protein), trypsinase (1 µg), or trypsin (1 µg) in 50 mM Tris-HCl, pH 8.0, in a volume of 100 µl for 1 h. After adding a dye, the reaction mixture was electrophoresed on a 20% SDS-polyacrylamide gel (Wako Pure Chemical Industries). Because the reaction product was too small to be separated by electrophoresis, the gel was heat-treated at 100°C for 1 h was used.

Behavioral Experiments. The day before the experiments were conducted, hair was removed from the rostral part of the back or the unilateral cheek of the mice using hair clippers. The animals were put individually in an acrylic cage composed of four cells (13 × 9 × 35 cm) for at least 1 h for acclimation. Intradermal injection was administered in a volume of 50 µl to the rostral back and 20 µl to the cheek. Immediately after intradermal injection, the animals were returned to the same cells, and their behaviors were videotaped for 1 h; no personnel were present in the observation room during this time. Playback of the video served for determination of hind-paw scratching of the rostral back or cheek and forelimb wiping of the cheek (Kuraishi et al., 1995; Shimada and LaMotte, 2008). When mice scratch, they stretch the hind paw toward the treated site, lean the head toward the hind paw, rapidly move the paw several times, and then lower it back to the floor; a series of these movements was counted as one bout of scratching (Andoh et al., 2004).

Determination of Trypsin-Like Serine Proteinase Activity. N-p-Tosyl-Gly-Pro-Arg p-nitroanilide acetate (Sigma-Aldrich), a substrate for trypsin-like serine proteases, was dissolved in 50 mM Tris-HCl, pH 8.0, in a concentration of 0.5 mg/ml. A 0.02-ml volume of ADV extract or heat-treated ADV extract was added to 0.18 ml of the substrate solution, and the mixture was incubated at 37°C for 1 h. The amount of p-nitroanilide released was colorimetrically determined at 420 nm.

Fig. 1.Scratching response to intradermal injection of dermatophyte extract into the back in ICR mice. Mice were administered an intradermal injection of an extract of the dermatophyte ADV or vehicle (VH). A, time course of scratching after ADV extract (20 µg/site) injection. B, dose-response curve for the scratch-inducing effect of ADV extract. Values represent the means ± S.E.M. for 8 to 14 animals. *, P < 0.05 compared with VH (Dunnett’s multiple comparisons). C, effect of heat treatment on the scratch-inducing activity of ADV extract. Heat-treated and untreated ADV extracts were injected intradermally at a dose of 20 µg/site. The dotted line represents the average value of the VH-injected group. Values represent the means ± S.E.M. for seven to eight animals. *, P < 0.05 (Student’s t-test).

Materials and Methods

Animals. Male ICR mice (5–9 weeks old or neonatal) were used, with the exception of one series of experiments in which male mast cell-deficient mice (WBB6F1 W/W) and the normal littermates [WBB6F1 (+/+)] were used at 8 weeks of age. All mice were purchased from Japan SL]
Fig. 2. Scratching and wiping responses to intradermal injection of dermatophyte extract into the cheek in ICR mice. Mice were administered an intradermal injection of ADV extract (20 µg/site) or VH, and scratching bouts and wiping actions of each mouse were counted for 1 h. Values represent the means ± S.E.M. for six animals. *, P < 0.05 (Student’s t test).

Fig. 3. Effects of opioid receptor antagonists on scratching responses to intradermal injection of dermatophyte extract into the back in ICR mice. Mice were administered an intradermal injection of ADV extract (20 µg/site), and scratching bouts were counted for 1 h. The opioid antagonist naltrexone hydrochloride, µ-opioid receptor antagonist naltrexone hydrochloride, and vehicle (VH) were injected subcutaneously 15 min before ADV extract injection. The dotted line represents the average value of scratching bouts in mice given intradermal injection of saline. Values represent the means ± S.E.M. for six animals. *, P < 0.05 compared with VH (Dunnnett’s multiple comparisons).

was stained with Coomassie Brilliant Blue (Wako Pure Chemical Industries), and the substrate peptide was determined.

Data Processing. Data are presented as means ± S.E.M. Statistical significance was analyzed using Dunnett’s multiple comparisons, Bonferroni’s multiple comparisons, or Student’s t test; P < 0.05 was considered significant.

Results

Behavioral Effects of Dermatophyte Extract. Trichophyton mentagrophytes is a common dermatophyte isolated from humans (Seebacher et al., 2008), and ADV, which belongs to the T. mentagrophytes complex, infects animals and humans (Drouot et al., 2009). Therefore, we examined the pruritogenic activity of an extract prepared from cultured ADV. When injected intradermally into the rostral back of mice, ADV extract elicited hind-paw scratching—an itch-related behavior—of the injection site at a dose of 20 µg/site; the effect peaked during the first 10-min period and almost subsided by 40 min (Fig. 1A). Scratching was dose-dependently increased in the range of 1 to 20 µg of ADV extract per injection site; significant increase was observed at the dose of 20 µg/site (Fig. 1B). Heat treatment of the ADV extract almost abolished its scratch-eliciting activity (Fig. 1C).

We also injected ADV extract into the murine cheek to test whether the extract is algogenic. Forelimb wiping—a nociceptive behavior—was slightly but significantly increased by ADV extract (20 µg/site) compared with the vehicle, whereas hind-paw scratching was markedly increased in the same individuals (Fig. 2); the increases in scratching elicited from injection into the cheek were similar to those from injection into the rostral back (Figs. 1B and 2).

Effects of Various Agents on ADV Extract-Induced Scratching. Subcutaneous pretreatment with the opioid receptor antagonist naloxone hydrochloride (1 and 10 mg/kg) and selective µ-opioid receptor antagonist naltrexone hydrochloride (1 and 10 mg/kg) inhibited ADV extract-induced scratching in a dose-dependent manner (Fig. 3). Oral pretreatment with 30 mg/kg H1 histamine receptor antagonist terfenadine had no effect (Fig. 4A). Intravenous pretreatment with the serine proteinase inhibitor nafamostat mesilate (1–10 mg/kg) inhibited ADV extract-induced scratching;

Fig. 4. Effects of H1 histamine receptor antagonist, serine protease inhibitor, and PAR2 antagonist on scratching responses to intradermal injection of dermatophyte extract into the back in ICR mice. Mice were administered an intradermal injection of ADV extract (20 µg/site), and scratching bouts were counted for 1 h. A, terfenadine (30 mg/kg) and VH were injected intraperitoneally together with ADV extract. Dotted lines represent the average value of scratching bouts in mice given intradermal injection of saline. Values represent the means ± S.E.M. for six animals. *, P < 0.05 compared with VH (Dunnnett’s multiple comparisons).

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significant inhibition was observed at a dose of 10 mg/kg (Fig. 4B). Simultaneous local treatment with the PAR2 receptor antagonist FSLRRY-NH$_2$ (10–100 μg/site) inhibited ADV extract-induced scratching, with a significant inhibition observed at a dose of 100 μg/site (Fig. 4C).

**Effect of Mast Cell Deficiency on ADV Extract-Induced Scratching.** An intradermal injection of ADV extract (20 μg/site) significantly increased scratching in mast cell-deficient mice (WBB6F1+/+ W/W$^v$) compared with saline-injected group (Fig. 5). The extent of ADV extract-induced scratching was similar in these mice (Fig. 5). An intradermal injection of the PAR2 receptor agonist SLIGRL-NH$_2$ (50 nmol/site) also significantly increased in WBB6F1+/+ W/W$^v$ and WBB6F1(+/+) mice, compared with negative control (50 nmol/site of the reverse peptide LRGILS-NH$_2$); the extent of SLIGRL-NH$_2$-induced scratching was similar in these mice (Fig. 6).

**Trypsin-Like Serine Protease Activity of ADV Extract.** The ADV extract (0.5–100 μg/ml) showed serine protease activity in a concentration-dependent manner (Fig. 7). Heat treatment markedly decreased the protease activity of the ADV extract with traces of activity remaining (Fig. 7).

**Cleavage of N-terminal Peptide of PAR2 Receptor by ADV Extract.** Proteolytic cleavage within the extracellular N terminus of PAR2 receptor exposes a receptor-activating N-terminal sequence that serves as a tethered ligand for the receptor (Macfarlane et al., 2001). A synthetic N-terminal peptide of PAR2 receptor, GRNNSKGRSLIGRLET-NH$_2$, was cleaved by the ADV extract as well as by trypsin and trypsin; thus, it disappeared from the reaction mixture after a 1-h reaction (Fig. 8A). In contrast, trypsin did not cleave the analog peptide GRNNSKHLILIGRLET-NH$_2$ (Fig. 8B). ADV extract decreased the analog peptide, but 63% remained after a 1-h reaction (Fig. 8B).

**Discussion**

Intradermal injections of ADV extract into the rostral back and cheek induced hind-paw scratching in mice, and its injection into the cheek elicited only slight wiping. Intradermal injections of pruritogenic and algogenic substances (such as histamine and capsaicin, respectively) into the cheek have been shown to elicit hind-paw scratching and forelimb wiping, respectively, of the injection site in mice (Shimada and LaMotte, 2008). Therefore, the present results suggest that the ADV extract is more pruritogenic and less algogenic. Itch-related, but not pain-related, behaviors are suppressed by opioid receptor antagonists (Akiyama et al., 2010; Gotoh et al., 2011). Opioid receptor antagonists have been shown to inhibit the scratching induced by several pruritogens (Andoh et al., 1998, 2009; Yamaguchi et al., 1999), dermatoses in rodents (Ohtsuka et al., 2001; Yamaguchi et al., 2001; Miyamoto et al., 2002), and pruritus in humans with pruritic diseases (Monroe, 1989; Bergasa et al., 1995). Opioid receptor antagonists exert antipruritic activity via the action on μ-opioid receptors in the central nervous system (Maekawa et al., 2002; Nojima et al., 2003), especially in the lower brainstem (Kuraishi et al., 2008). Thus, the result that the scratching induced by ADV extract injection into the rostral back was suppressed by opioid receptor antagonists supports the idea that ADV extract was primarily pruritogenic in the skin.

ADV extract-induced scratching was not inhibited by the H$_1$ histamine receptor antagonist terfenadine, even at a dose that almost completely inhibits both histamine-induced scratching (Ohtsuka et al., 2001) and immediate allergic-induced plasma extravasation (Ohtsuka et al., 2001; Andoh et al., 2011).
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Kuraishi, unpublished observation). Thus, dermatophyte keratinase may be pruritogenic, but we do not deny the possibility that the other dermatophyte proteases are also pruritogenic.

PAR₂ receptor is activated via the proteolytic cleavage of its N-terminal sequence by serine proteases (Macfarlane et al., 2001). In the present study, the ADV extract as well as tryptase and trypsin cleaved the N-terminal peptide of PAR₂ receptor, suggesting that the ADV extract has PAR₂-stimulating activity. The PAR₂ receptor antagonist FSLLRY-NH₂ (Al-Ani et al., 2002) inhibited the ADV extract-induced scratching. The dose response of the anti-ADV effect of FSLLRY-NH₂ was similar to that of its effect on scratching induced by intradermal tryptase in mice (Ui et al., 2006). Thus, it is suggested that the ADV extract caused scratching via the activation of PAR₂ receptors. The activation of PAR₁ and PAR₄ also causes scratching, at least partly through the release of histamine from mast cells (Tsujii et al., 2008). However, as mentioned above, histamine and mast cells did not play essential roles in the scratch-inducing activity of ADV extract, and PAR₁ and PAR₄ receptors may not be involved in the ADV action. Tryptase almost completely cleaved an N-terminal peptide of PAR₂ receptor, GRNNSKGRLIGRLET-NH₂, but not the analog GRNNSKGIIILGRLET-NH₂, suggesting that the analog peptide is resistant to trypsin-like serine protease. The ADV extract almost completely or partly cleaved the N-terminal peptide of PAR₂ receptor or the analog, suggesting that although the ADV extract has mainly trypsin-like serine protease activity, it also has other protease activity. It is unknown whether non-trypsin like protease activity is involved in the scratch-inducing action of the ADV extract.

Because dermatophytes generally invade only keratinized structures, the epidermis may be a causative site for dermatophytosis pruritus. The PAR₂ receptors are present in a high density in epidermal keratinocytes (Steinhoff et al., 2003; Tsujii et al., 2009), except in the basal layer (Tsujii et al., 2009). Keratinocytes release several itch mediators and itch enhancers, such as leukotriene B₄ (Andoh and Kuraishi, 1998; Andoh et al., 2001, 2004, 2009), thromboxane A₂ (Andoh et al., 2007), and nitric oxide (Andoh and Kuraishi, 2009). Keratinocytes release several itch mediators and itch enhancers, such as leukotriene B₄ (Andoh and Kuraishi, 1998; Andoh et al., 2001, 2004, 2009), thromboxane A₂ (Andoh et al., 2007), and nitric oxide (Andoh and Kuraishi, 2009). Recently, it has been shown that leukotriene B₄ is produced in cultured keratinocytes by stimulation of PAR₂ receptors and that intradermal PAR₂ agonist-induced scratching is suppressed by a 5-lipoxygenase inhibitor in mice (Zhu et al., 2009). These findings taken together raise the possibility that serine proteases secreted by dermatophytes activate PAR₂ receptors in the epidermal keratinocytes to secrete itch mediators including leukotriene B₄.

PAR₂ receptors are also present in nerve fibers in the human skin (Steinhoff et al., 2003). In rodents, PAR₂ receptors are expressed in neurons in the dorsal root ganglion, and some PAR₂-positive neurons contain neuropeptides such as substance P and calcitonin gene-related peptide (Steinhoff et al., 2000). It has been reported that intradermal trypsin-induced scratching is mediated by substance P release and mast cell degranulation, namely mediated by a neurogenic inflammatory mechanism, in mice (Costa et al., 2008). However, in the present study, an intradermal injection of ADV extract increased scratching in both mast cell-deficient mice and normal littermates, thus, excluding the contribution of mast cell in the response. In addition, an intradermal injec-

![Fig. 8](https://jpet.aspetjournals.org)
tion of PAR2 receptor agonist peptide increased scratching in both mast cell-deficient mice and normal littermates; the extent of scratching was similar to that in ICR mice (Tsujii et al., 2009). In preliminary experiments, the NK1 tachykinin receptor antagonists spantide N2-[(4R)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl]-L-tryprol-N-phenylmethyl-3-(2-naphthyl)-l-alaninamide (FK888) (Fujii et al., 1992) did not inhibit ADV extract-induced scratching (data not shown). Thus, our data suggest that neurogenic inflammation does not play a key role in ADV extract-induced and PAR2-mediated scratching. It is conceivable that serine proteases from dermatophytes act directly on the pruriceptive primary afferents. However, PAR2-immunoreactive nerve-like structures have not been observed in the skin, including the dermis just beneath the epidermis in mice (Tsujii et al., 2009). Thus, further studies are needed to elucidate the direct action of serine proteases on primary afferents.

In summary, our data suggest that serine proteases secreted by dermatophytes cause itching through activation of the PAR2 receptors, which may be a causal mechanism of dermatophytosis itch.

Authorship Contributions
Participated in research design: Andoh, Sano, and Kuraishi.
Conducted experiments: Andoh, Takayama, and Yamashiko.
Contributed new reagents or analytic tools: Lee and Sano.
Performed data analysis: Andoh, Takayama, Yamashiko, Shimizu, and Kuraishi.
Wrote or contributed to the writing of the manuscript: Andoh and Kuraishi.

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