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

Tsugunobu Andoh, Yusuke Takayama, Takako Yamakoshi, Jung-Bum Lee, Ayako Sano, Tadamichi Shimizu, and Yasushi Kuraishi

Departments of Applied Pharmacology (T.A., Y.T., Y.K.), Dermatology (T.Y., T.S.), and Pharmacognosy (J.-B.L.), Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan; and Graduate School of Agriculture, University of the Ryukyu, Okinawa, Japan (A.S.)

Received April 4, 2012; accepted July 2, 2012

ABSTRACT

We investigated the involvement of serine protease and proteinase-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 scratching 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 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 pruritus 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 proteinases, cause acute itching. Proteinases have long been known to cause itching in humans; moreover, endopeptidases rather than exopeptidases cause itching (Arthur and Shelley, 1955). Among the endopeptidases (proteases), serine proteinases 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 terminus, which activates PAR itself (Macfarlane et al., 2001). The PAR1, PAR3, and PAR4 receptor subtypes are thrombin receptors, whereas PAR2 is activated by trypsin-type serine proteinases rather than by thrombin (Macfarlane et al., 2001). Trypsin-type serine proteinases and a synthetic PAR2 tethered ligand cause itching and scratching in humans and animals (Steinhoff et al., 2003; Shimada et al., 2006; Ui et al., 2006; Tsujii et al., 2009). Chymase, a chymotrypsin-type serine proteinase, causes itching in humans, probably by degranulating mast cells (Hagermark et al., 1972). Therefore, the second aim of this study was to determine whether the PAR2 receptor and mast cell degran-
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 5% glucose (Wako Pure Chemical Industries) and injected intravenously 5 min before ADV extract injection. The peptides FSLLRY-NH₂, SLIGRL-NH₂, LRGILS-NH₂, GRNNSKGRSLIGRLT-NH₂, and LIGRLET-NH₂ (an N-terminal peptide of PAR2 containing a tethered ligand sequence SLIGRL) and its analog GRNNSKGRSLIGRTEL-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), trypstatine (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 products were too small to be separated by electrophoresis, the gel

**Dermatophytes and Extract Preparation.** The dermatophyte ADV was obtained from the National BioResource Project (http://www.nbrp.jp/). It was subcultured on 2% agar (Wako Pure Chemical Industries) containing potato dextrose broth (Wako Pure Chemical Industries) at room temperature. A colony containing fungal spores was removed, added to Sabouraud dextrose liquid culture medium containing 2% dextrose (Wako Pure Chemical Industries) and 1% polypeptone (Wako Pure Chemical Industries), and incubated at 37°C for more than 3 days. The colonies of ADV were repeatedly washed with physiological saline and centrifuged until the supernatant became transparent. The resultant pellet was suspended in physiological saline and subcutaneously injected 1 h before ADV extract injection. The peptides FSLLRY-NH₂, SLIGRL-NH₂, LRGILS-NH₂, GRNNSKGRSLIGRLET-NH₂, and GRNNSKGRSLIGRTEL-NH₂ were synthesized and identified using the peptide synthesizer FSSM-8 (Shimazu Co., Kyoto, Japan) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer AutoFlex T1 (Bruker Daltonics, Billerica, MA), respectively. FSLLRY-NH₂ was dissolved in physiological saline (Ohtsuka Pharmaceutical Co., Ltd., Tokushima, Japan) and injected intradermally together with ADV extract. 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.

**Activity of PAR₂ Cleavage.** Ten micrograms of GRNNSKGRSLIGRLET-NH₂ (an N-terminal peptide of PAR₂ containing a tethered ligand sequence SLIGRL) and its analog GRNNSKGRSLIGRTEL-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), trypstatine (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

**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).
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 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 protease inhibitor nafamostat mesylate (1–10 mg/kg) inhibited ADV extract-induced scratching;
significant inhibition was observed at a dose of 10 mg/kg (Fig. 4B). Simultaneous local treatment with the PAR2 receptor agonist FSLRR-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$^*$) and in normal littermates ([WBB6F1(+/+)], 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$^*$ 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, GRNNSKGRLIGRET-NH$_2$ (Fig. 8A), 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 GRNNSGKILIGRET-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 wip-

![Fig. 5. Effect of mast cell deficiency on scratching responses to intradermal injection of dermatophyte extract into the back. ADV extract (20 µg/site) and vehicle (VH) were injected intradermally in mast cell-deficient WBB6F1 W/W$^*$ mice and in normal littermates (WBB6F1(+/+)). Scratch-inducing effect of PAR2 receptor agonist peptide in WBB6F1 mice. The PAR2 receptor agonist peptide SLIGRL-NH$_2$ (SLIGRL) and the reverse peptide (RP) LRGILS-NH$_2$ were injected intradermally at a dose of 50 nmol/site in mast cell-deficient WBB6F1 W/W$^*$ mice and in normal littermates (WBB6F1(+/+)). Scratching bouts were counted for 1 h after intradermal injection. Values represent the means ± S.E.M. for seven (SLIGRL) or eight (RP) animals. *, $P < 0.05$ compared with the corresponding RP (Bonferroni’s multiple comparisons).](image1)

![Fig. 6. Scratch-inducing effect of PAR$_2$ receptor agonist peptide in WBB6F1 mice. The PAR$_2$ receptor agonist peptide SLIGRL-NH$_2$ (SLIGRL) and the reverse peptide (RP) LRGILS-NH$_2$ were injected intradermally at a dose of 50 nmol/site in mast cell-deficient WBB6F1 W/W$^*$ mice and in normal littermates (WBB6F1(+/+)). Scratching bouts were counted for 1 h after intradermal injection. Values represent the means ± S.E.M. for seven (SLIGRL) or eight (RP) animals. *, $P < 0.05$ compared with the corresponding RP (Bonferroni’s multiple comparisons).](image2)

![Fig. 7. Trypsin-like serine protease activity in the dermatophyte extract. ADV extract with or without prior heat treatment were added to the solution of N-p-Tosyl-Gly-Pro-Arg p-nitroanilide, a substrate for trypsin-like serine proteases. The amount of p-nitroanilide released was colorimetrically determined. Values represent the means ± S.E.M. for eight samples. *, $P < 0.05$ compared with VH (Dunnett’s multiple comparisons).](image3)
Matophyte extract, tryptase, and trypsin. A, GRNNSKGRSLIGRLET-
induced scratching in mice (T. Andoh, Y. Takayama, and Y. et al., 2010). In addition, ADV extract elicited scratching to a
similar extent in both mast cell-deficient mice and normal littermates. Therefore, it is suggested that histamine and mast cell degranulation are not the main causes of ADV-induced scratching.

The ADV extract had serine protease activity and its scratch-inducing activity was suppressed by nafamostat mesylate, a serine protease inhibitor (Mori et al., 2003). The dose response of the anti-ADV effect of nafamostat was similar to that of its effect on scratching induced by intradermal trypsin in mice (Ui et al., 2006). Taken together, these results suggest that serine proteases are involved in ADV extract-induced itching. The results that heat treatment of the ADV extract markedly decreased its scratch-inducing and serine protease activities support the above-mentioned idea. Dermatophytes require keratin for growth and generally invade only superficial 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, 2003). 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-
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 I and II (Fujii et al., 2008). In preliminary experiments, the NK1 tachykinin receptor antagonists spantide I and II (Fujii et al., 2008) 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.

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

References
Andoh, Takayama, Yamakoshi, Shimizu, and Kuraishi.
Address correspondence to: Yasushi Kuraishi, Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-1094, Japan. E-mail: kuraisiy@pha.u-toyama.ac.jp