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Tryptase and protease-activated receptor-2 stimulate scratching behavior in a murine model of ovalbumin-induced atopic-like dermatitis

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ABSTRACT

The aim of the current study was to investigate the involvement of tryptase and protease-activated receptor-2 (PAR2) in the pathogenesis of itch using a recently developed murine model of atopic dermatitis (AD) elicited by epicutaneous sensitization with ovalbumin (OVA). We also examined whether tacrolimus exerts an antipruritic effect. Epicutaneous sensitization of BALB/c mice with OVA led to a significant increase in the number of scratches. Notably, PAR2 mRNA and protein levels as well as cutaneous levels of tryptase were significantly enhanced in epicutaneously sensitized mice. Pretreatment with the protease inhibitor, leupeptin, PAR2 antibody, and tacrolimus significantly reduced the number of degranulated mast cells and tryptase content, and consequently alleviated scratching behavior. Cetirizine (10 mg/kg) exerted a significant inhibitory effect on the scratching behavior of mice, but did not affect the number of degranulated mast cells and induction of tryptase. Our results collectively suggest that tryptase and PAR2 are involved in OVA allergy-induced scratching behavior.
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1. Introduction

Severe itching is a common phenomenon in atopic dermatitis (AD), which is poorly controlled in the clinic and has a major effect on quality of life [1,2]. The molecular basis of pruritus in AD remains largely unexplained at present. Histamine is proposed as one of the main mediators of itch transmission and histamine H_1 receptor antagonists are widely used for pruritus in patients with AD. Protease-activated receptor-2 (PAR2) belongs to a recently described G protein-coupled receptor subfamily activated by serine proteinases. PAR2 is suggested to mediate widespread inflammation in various tissues, including skin [3,4]. Recently, Steinhoff et al. [5] reported that PAR2 regulates cutaneous inflammation via a neurogenic mechanism. Pathogen- and environment-derived proteases, as well as tryptase released endogenously by mast cells, activate PAR2 localized in C nerve fiber terminals in the skin. PAR2 has been shown to play a key role in the pathophysiology of itch [6,7]. A previous study demonstrated that a histamine H_1 receptor antagonist has little effect on PAR2-activating peptide-induced scratching behavior in mice, indicating that PAR2 is a histamine-independent mediator of itch [8].

However, the precise mechanism underlying PAR2-mediated itch during inflammatory processes is poorly understood at present and requires more detailed investigation in animal models. Spergel and co-workers described a murine model of AD elicited by repeated epicutaneous sensitization with ovalbumin (OVA), which displays several features of human AD, including elevated total and specific IgE, dermatitis characterized by infiltration of $CD3^+$ T cells and eosinophils into the dermis, and increased local mRNA expression of TH2 cytokines [9,10]. Yatsuzuka et al. [11] further demonstrated that application of OVA results in significantly increased scratching behavior, and endogenous mediators other than histamine may be responsible for provoking the itch sensation during the last stage. In the present study, we aimed to examine PAR2 expression using the above mouse AD model and assess its specific role in the pathogenesis of itch. Additionally, in view of data obtained from recent clinical trials showing that short-term

Abbreviations: AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HPF, high power fields; KLKs, kallikrein-related peptidases; IgE, immunoglobulin E; OVA, ovalbumin; PAR2, protease-activated receptor-2.

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administration of topical tacrolimus is effective and safe in controlling pruritus and skin inflammation [12], we examined whether tacrolimus produced an antipruritic effect in a murine model.

2. Materials and methods

2.1. Experimental animals

Female BALB/c mice (4–6-weeks of age) obtained from SIBS, China, were kept in a pathogen-free environment with a temperature of 22 ± 1 °C and a humidity of 60 ± 5%. All procedures with mice were performed in accordance with the Guidelines for Animal Experiments of the Second Military Medical University, Shanghai, China.

2.2. Drugs and reagents

The effects of drugs on scratching behavior were evaluated immediately after removal of the patch in mice after systemic immunization. A histamine H_1 receptor antagonist, cetirizine (Lianhuan Pharma, Jiangsu, China), was dissolved in physiologic saline at various concentrations, and administered orally 1 h before measurement of scratching behavior. The serine protease inhibitor, leupeptin (Sigma, St. Louis, MO, USA), was dissolved in physiologic saline at various concentrations and injected intraperitoneally into mice 30 min before the measurement of scratching behavior. Anti-PAR2 immunoglobulin G antibody (Santa Cruz, CA, USA) and nonspecific IgG (Santa Cruz, CA, USA) were dissolved in phosphate-buffered saline (PBS, pH 7.4) and injected intradermally 30 min before the measurement of scratching behavior. Tacrolimus (FK506; Qiao Chemical, Shanghai, China) was dissolved in 70% of ethanol at various concentrations (*w*/*v*) and administered topically 1 h before the assessment of scratching behavior.

2.3. Sensitization

Epicutaneous sensitization of anesthetized mice was performed as described by Spergel et al. [9]. In brief, hair over the rostral part of the back of each mouse was shaved at 2-week intervals. OVA (100 g, SIBS, China) in 100 μl of normal saline solution or placebo (100 μl of normal saline solution) was applied to a 1 × 1 cm patch of sterile gauze. To prevent mice scratching off the gauze, the gauze was secured to the rostral part of the back with transparent bioocclusive dressing (5 × 1 cm, Johnson & Johnson Medical Inc., USA). Patches were placed for a 1-week period and subsequently removed. Two weeks later, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1-week exposures to the patch separated by 2-week intervals.

2.4. Behavioral observations

Scratching behavior was evaluated according to the method of Kuraishi et al. [13]. Before the experiment, mice were placed in an observation cage (18 cm × 24 cm × 30 cm) composed of four cells for 1 h of acclimation. Immediately after removal of the patch, mice were placed back into the observation cage and their scratching behavior was recorded automatically using an 8 mm video camera (CCD-700 V, Sony, Tokyo, Japan) for 1 h with no-one present in the observation room. Scratching frequency was established by replaying the recorded videotapes. Scratching of the rostral part of the back with hindpaws was counted as an itch response. One scratching bout generally consisted of more than three repetitions of hindpaw scratching movements.

2.5. RT-PCR

Skin biopsies were obtained 24 h after the third patch was removed and immediately frozen in liquid nitrogen. Total RNA extraction was performed using the TRIzol method (Gibco Canada). The assay was performed according to the manufacturer's protocol. RNA was reverse-transcribed and PAR2 DNA amplified using the cycle conditions described previously [14]. The gel was scanned under UV light, and bands quantified using a GeneGenius gel documentation and analysis system (ABI-7300, USA). The amount of each mRNA transcript was normalized with that of GAPDH mRNA.

2.6. Western blotting analysis

Skin biopsies were obtained and frozen as described above. PAR2 protein expression was analyzed using western blotting, as described previously [15]. Densitometric analysis of bands on developed X-ray films was performed using Smartview image software (Shanghai Furi Science & Technology Co., LTD, China). All data were normalized to β-actin.

2.7. Histological analyses

For histological examination, specimens were obtained from patch areas 24 h after removal of the patch from the third sensitization. Specimens were fixed with 10% neutral buffered formalin and embedded in white paraffin. Serial 5 μm paraffin sections were prepared and stained with hematoxylin and eosin (H&E). Mast cells were identified by staining slides with toluidine blue, quantified by counting in 10–20 high power fields (HPF), and expressed as mast cells/HPF. The percentage of degranulated mast cells was calculated.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Skin biopsies were obtained and frozen as described above. Treated skin was minced and homogenized in ice-cold PBS with a Polytron tissue homogenizer on ice. The precipitate was removed by centrifugation at 1000 ×*g* for 20 min at 4 °C. The supernatant was freeze-dried and resuspended in enzyme immunoassay buffer. Tryptase concentration was determined using a specific enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc, Wuhan, China), according to the manufacturer's instructions.

2.9. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Data were analyzed using Student's t-test and Dunnett's test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of chemicals on scratching behavior in BALB/c mice elicited by repeated epicutaneous sensitization with OVA

Epicutaneous sensitization with OVA led to a significant increase in the number of scratches in BALB/c mice, compared to that in the saline group. Cetirizine (10 mg/kg) induced a marked inhibition in OVA-induced scratching behavior, but had no significant effects at doses of 1 and 3 mg/kg. Leupeptin (5, 10, and 20 mg/kg) significantly suppressed OVA-induced scratching behavior in a dose-dependent manner. PAR2-neutralizing antibody (at doses of 0.1 and 1.0 μg/site) also inhibited OVA-induced scratching behavior to a significant extent. Non-specific IgG had no effect on scratching behavior at a dose of 1.0 μg/site. In contrast, topical application of tacrolimus suppressed OVA-induced scratching behavior in a dose-dependent manner, with significant effects at concentrations of 1.0%, 3.0% and 10.0%, as shown in Fig. 1.

3.2. PAR2 mRNA and protein expression in BALB/c mice subjected to repeated epicutaneous sensitization with OVA

Expression of PAR2 in skin was assessed using RT-PCR and western blotting analyses. After epicutaneous sensitization with OVA, high PAR2 expression was observed in lesional skin biopsies. RT-PCR analysis

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