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# Involvement of chymase in allergic conjunctivitis of guinea pigs

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ABSTRACT

It has been reported that chymase activity was increased in allergic conjunctivitis patients and this activity was correlated with the severity of the disease. However, the precise roles of chymase in allergic conjunctivitis are unclear, and whether chymase inhibitors are effective for allergic conjunctivitis has not been reported even in experimental animal models. In this study, the roles of chymase in the pathogenesis were evaluated using a selective chymase inhibitor, ONO-WH-236, in a guinea pig model of allergic conjunctivitis induced by cedar pollen. Sensitized guinea pigs were challenged by the pollen, followed by assessing redness and edema in the conjuntiva, and counting the frequency of eye scratching as an itch-associated response. Treatment with the ONO-WH-236 (40 and 80 mg/kg, p.o.) dose-dependently inhibited the induction of redness, edema and scratching behavior. An anti-histaminic drug, ketotifen (3 mg/kg, p.o.), also significantly inhibited conjunctivitis symptoms. Chymase activity was increased in ophthalmic lavage fluid immediately after the pollen challenge. The increase in chymase activity was inhibited by in vivo treatment with ONO-WH-236. Interestingly, increased histamine in the ophthalmic lavage fluid immediately after the challenge was also inhibited by the chymase inhibitor. Administration of human recombinant chymase by eye dropping (0.09 and 0.9 μg/eye) dose-dependently induced scratching behavior, which was inhibited by not only ONO-WH-236 but also ketotifen; however, chymase administration induced only weak redness in the conjunctiva, which was resistant to treatment with anti-histaminic drugs. In conclusion, it was suggested that chymase was released from mast cells after antigen challenge, followed by the induction of conjunctivitis symptoms through histamine release from mast cells. Thus, chymase could be a potential target for pharmacotherapy for allergic conjunctivitis.



## 1. Introduction

Mast cells in human tissues are classified into 2 types, $MC_{TC}$ expressing tryptase, chymase and carboxypeptidase A, and $MC_T$ expressing only tryptase (Caughey, 2007; Stevens and Adachi, 2007; Pejler et al., 2010). Among the mast cell proteases, chymase has the characteristic feature of being a chymotrypsin-like serine protease, which is present in the secretory granules of mast cells. It is generally known that upon anaphylactic stimulation, mast cells release chymase together with co-stored proteases, tryptase and carboxypeptidase A, as well as a pre-formed mediator, histamine. Regarding pro-inflammatory functions, chymase can be a chemical mediator that induces the recruitment of inflammatory cells because intradermal injection of human chymase caused the accumulation of neutrophils and eosinophils in guinea pigs (He and Walls, 1998). This chemoattractant activity of chymase was also confirmed in vitro experiments (Tani et al., 2000; Terakawa et al., 2006). In addition, it has been reported that chymase activated pro-interleukin (IL)-1β to IL-1β (Mizutani et al., 1991), and pro-IL-18 to IL-18 (Omoto et al., 2006), suggesting that chymase could indirectly promote inflammatory responses. Furthermore, interactions between chymase and histamine in anaphylactic responses in mast cells have been demonstrated. It has been reported that anaphylactic histamine release from human and rat mast cells was effectively suppressed by suppression of chymase activity (Katunuma et al., 1986; He et al., 1999, 2004). To date, chymase inhibitors have been developed, and assessed in various models of diseases. In

*Abbreviations:* AMC, 7-amino-4-methylcoumarin; CIS, conjuctivitis intensity score; DMSO, dimethyl sulfoxide; IL, interleukin; $MC_T$, tryptase-positive mast cells; $MC_{TC}$, tryptase-positive, chymase-positive mast cells; NO, nitric oxide; NOS, nitric oxide synthase; OLF, ophthalmic lavage fluid; PAR, protease-activated receptor.

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chronic atopic dermatitis models, chymase inhibitors effectively prevented eosinophil accumulation, formation of edema in the skin, and the itch-associated scratching response (Watanabe et al., 2002; Terakawa et al., 2008).

On the other hand, in a typical immediate-type allergic disease, allergic conjunctivitis, chymase activity in the tears of vernal keratoconjunctivitis patients was significantly higher than in healthy subjects (Ebihara et al., 2004). The level of chymase activity correlated well with the severity of the disease (Ebihara et al., 2004). The predominant type of mast cells in the substantia propria of human conjunctival tissue was $MC_{TC}$ (Irani et al., 1990). In addition, rat mast cell chymase elicited itch in human skin (Hägermark et al., 1972). Andoh et al. (2012) indicated that allergic itch induced by ragweed pollen in sensitized mice was completely dependent on mast cells, whereas involvement of histamine in it was partial. Thus, it has been strongly suggested that chymase is involved in the pathogenesis of allergic conjunctivitis, and that inhibition of chymase can be a therapeutic strategy for the disease. However, there has been no report demonstrating the efficacy of chymase inhibitors on allergic conjunctivitis even in experimental animal models, and the pathophysiological roles of chymase in allergic conjunctivitis have been unclear.

In order to analyze the mechanisms underlying allergic conjunctivitis, we have developed and utilized a guinea pig model of Japanese cedar pollen-induced allergic conjunctivitis (Yasuda et al., 1999; Fukushima et al., 2003; Katayama et al., 2005; Nagata et al., 2008). In the experimental model, when sensitized guinea pigs were challenged by dropping pollen into the eyes once a week, histamine-dependent conjunctivitis symptoms, redness and edema in the conjunctiva, and itch-associated behavior were evoked in the 1st–3rd challenges (Fukushima et al., 2003). In this study, the effect of a chymase inhibitor, ONO-WH-236, on pollen-induced conjunctivitis was examined in comparison with an antihistaminic drug, ketotifen. In addition, interaction between chymase and histamine was assessed using exogenously applied chymase-induced conjunctivitis, and histamine levels were measured after allergen and chymase challenges. Consequently, we could obtain data indicating that chymase is involved in the induction of allergic conjunctivitis symptoms directly or indirectly through histamine release.

## 2. Materials and methods

### 2.1. Effect of chymase inhibitor on chymase activity in vitro

ONO-WH-236 (Ono Pharmaceutical Co. Ltd., Osaka, Japan) was used as the chymase inhibitor and its effect on human chymase activity was assessed. N-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (final concentration 2.5 mM; Sigma–Aldrich, St. Louis, MO, USA) was incubated with various concentrations of ONO-WH-236 or the vehicle, 5% (final concentration) dimethyl sulfoxide (DMSO) in an assay buffer, 50 mM Tris–HCl, 150 mM NaCl, 50 U/ml heparin (pH 7.6) for 10 min. After pre-incubation, human chymase (EastCoast Bio, Inc., North Berwick, ME, USA) was added at a final concentration of 0.2 μg/ml, and then liberated *p*-nitroaniline was measured by photometric determination at 405 nm for 5 min.

To assess the specificity of ONO-WH-236 on chymase activity, its effect on chymotrypsin activity was also evaluated. N-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (final concentration of 250 μM) was incubated with various concentrations of ONO-WH-236 or the vehicle, 5% DMSO in an assay buffer, 100 mM Tris–HCl, 10 mM $CaCl_2$ (pH 7.8) for 10 min. After pre-incubation, α-chymotrypsin from bovine pancreas (Sigma–Aldrich), which was dissolved in 2 mM HCl, was added to the mixture at a final concentration of 0.005 units/ml, followed by kinetic measurement of liberated *p*-nitroaniline by photometric determination at 405 nm for 5 min.

### 2.2. Animals

Male 3-week-old Hartley guinea pigs were purchased from Japan SLC (Hamamatsu, Japan). Animals were housed in an air-conditioned room at 22–24 °C and 50–70% humidity, with lights on from 08:00 to 20:00. Access to standard laboratory diet and water was provided *ad libitum*. Study protocols of allergic conjunctivitis in guinea pigs were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

### 2.3. Allergic conjunctivitis

As previously described (Yasuda et al., 1999; Fukushima et al., 2003; Katayama et al., 2005; Nagata et al., 2008), guinea pigs were sensitized by intraperitoneal injections with an extract of Japanese cedar pollen plus $Al(OH)_3$ (10 μg pollen protein and 20 mg $Al(OH)_3$/ml per animal) twice within a week. Two weeks after the second sensitization, animals were challenged by dropping a pollen suspension (2 mg pollen/10 μl/eye) without $Al(OH)_3$ into each of their eyes. The challenge was conducted once a week until the 3rd challenge.

### 2.4. Chymase-induced conjunctivitis

Chymase human recombinant (Sigma–Aldrich) was administered at doses of 0.09 and 0.9 μg/10 μl/eye by dropping into both eyes of sensitized guinea pigs on the 7th day after the 2nd or 3rd challenges. These doses of human chymase were comparable to those in another guinea pig study (He and Walls, 1998), in which 0.25 and 2.5 μg/site of human chymase was intradermally injected into guinea pigs.

### 2.5. Evaluation of conjunctivitis

The magnitude of conjunctival redness and edema was macroscopically judged and expressed as a conjunctivitis intensity score (CIS) according to an arbitrary 5-point graded scale, in which a score of 0 indicates no symptoms; 1, light symptoms; 2, mild symptoms; 3, moderate symptoms; and 4, severe symptoms (Yasuda et al., 1999). Additionally, symptoms falling between scores 0 and 1 were judged as score 0.5.

Scratching frequencies at 0–0.5 and 0.5–1 h after either pollen or chymase challenge were counted. The scratch response was defined as an uninterrupted cluster of rapid hind limb movements that were precisely directed to the ocular surface.

### 2.6. Treatment with chymase inhibitor and anti-histaminic drug

The chymase inhibitor, ONO-WH-236 (40 and 80 mg/kg) and an anti-histaminic drug, ketotifen (3 mg/kg; Sigma–Aldrich) were orally administered once, 1 h before the 2nd or 3rd pollen challenge. ONO-WH-236 and ketotifen were suspended and dissolved, respectively, in 0.5% methyl cellulose solution before the use. We have reported that inhibition by a classical anti-histaminic drug, pyrilamine, was consistently observed in the 1st-3rd challenges in an allergic conjunctivitis model (Fukushima et al., 2003). In the experiment of chymase-induced conjunctivitis, these compounds were orally administered 1 h before chymase application.

When ONO-WH-236 was once orally administered at a dose of 100 mg/kg in rats, bioavailability was 78%, and the $t_{1/2}$ was 3.7 h.