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# Eosinophil migration induced by mast cell chymase is mediated by extracellular signal-regulated kinase pathway

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**Abstract**

Mast cell chymase is known to induce eosinophil migration in vivo and in vitro. In the present study, we investigated possible involvement of mitogen-activated protein (MAP) kinases; extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38, in the chymase-induced eosinophil migration. Human chymase induced a rapid phosphorylation of ERK1/2 and p38 in human eosinophilic leukemia EoL-1 cells, while no phosphorylation was detected in JNK. The chymase-induced phosphorylation of ERK and p38 was inhibited by pertussis toxin. Similar results were obtained in the experiments using mouse chymase and eosinophils. U0126 (the inhibitor for MAP/ERK kinase) suppressed chymase-induced migration of EoL-1 cells and mouse eosinophils. However, SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) showed little effect on the migration. It is suggested therefore that chymase activates ERK and p38 probably through G-protein-coupled receptor, and that ERK but not p38 cascade may have a crucial role in chymase-induced migration of eosinophils.


*Keywords:* Chymase; Mast cells; Eosinophils; Chemotaxis; MAP kinase; Signal transduction

Chymase is a chymotrypsin-like serine protease exclusively stored in mast cells and secreted by the cells upon degranulation [1]. The physiological and pathological roles of chymase have not been fully elucidated, but this enzyme appears to participate in inflammation and allergic reaction, judging from its ability to hydrolyze a variety of cytokines [2–4] and extracellular matrixes [5]. It has recently been reported that intradermal injection of chymase causes accumulation of inflammatory cells such as eosinophils in the skin [6–8]. In addition, chymase stimulates migration of inflammatory cells in vitro [7–9], suggesting that chymase functions as a chemoattractant. Consistently, administration of chymase inhibitor reduced eosinophil accumulation in the dermis in chronic dermatitis induced by repeated epicutaneous application of 2,4-dinitrofluorobenzene [7].

The majority of chemoattractants are known to act through seven transmembrane, G-protein-coupled receptors [10], and binding of the chemoattractants to their receptors leads to a transient intracellular rise in $Ca^{2+}$ concentration [11]. In fact, human chymase causes intracellular $Ca^{2+}$ elevation, which is blocked by pretreatment with pertussis toxin, the G-protein-inhibitor [12]. This finding suggests an existence of receptor for chymase, which would resemble protease-activated receptors (PARs).

Mitogen-activated protein (MAP) kinases are important mediators of signal transduction and participate in multiple cellular functions, such as proliferation, differentiation, and locomotion [13,14]. In mammalian cells, there are three families of MAP kinases, i.e., extracellular signal-regulated kinase (ERK; ERK1 and ERK2),

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c-Jun amino-terminal kinase (JNK), and the p38 subgroup of kinases. These MAP kinases are regulated by phosphorylation cascades; two upstream protein kinases activated in series lead to activation of a MAP kinase [13–15]. The kinase immediately upstream of the MAP kinase is a member of MAP/ERK kinase (MEK) family, and MEKs are also activated by diverse MEK kinases.

In the present study, we have investigated the involvement of members of MAP kinase cascade in chymase-induced eosinophil chemotaxis using human eosinophilic leukemia cell line EoL-1 and mouse eosinophils. Our data indicate, for the first time, that ERK pathway is important for the chymase-induced signaling pathways in eosinophil migration.

## Materials and methods

*Reagents.* Human chymase was purchased from Sigma–Aldrich Japan (Tokyo, Japan). Recombinant mouse mast cell protease-4 (mMCP-4) was prepared as described in [8]. Pertussis toxin and MAP kinase inhibitors (U0126, SB203580, and SP600125) were purchased from Biomol (Plymouth Meeting, PA) and CN Biosciences (SanDiego, CA), respectively. Chymase inhibitor SUN C8257 was synthesized as described previously [16].

*Cell culture.* A human eosinophilic leukemia cell line EoL-1 [17] was obtained from Riken BioResource Center (Tsukuba, Japan) and maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 pg/ml streptomycin. Induction of differentiation of the cells into eosinophil-like cells with butyric acid was performed as described in [18]. The content of eosinophil-derived neurotoxin was measured using enzyme-linked immunosorbent assay (ELISA) kit for the protein (Biocompare, South San Francisco, CA).

*Isolation of mouse eosinophils.* Mouse eosinophils were isolated from the peritoneal fluid of the mice with allergen-induced peritonitis [19]. Briefly, BALB/c mice were immunized by subcutaneous injection with ragweed extract (1/1000 dilution, Torii Pharmaceutical, Ltd, Tokyo, Japan) on days 0, 1, 6, 8, and 14. Six days after the last immunization (on day 20), the mice were challenged by intraperitoneal injection of 0.2 ml ragweed extract (1/1000 dilution). Forty-eight hours after the elicitation, peritoneal cells were collected by peritoneal lavages with 2.0 ml of phosphate-buffered saline containing 6 U/ml heparin. Eosinophils used for chemotaxis analyses were purified by negative selection using Thy1.2 and B220 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Eosinophils used for phosphorylation of MAP kinases were further purified by fluorescence-activated cell sorting (FACS) with phycoerythrin-conjugated anti-CCR3 monoclonal antibodies (R&D Systems, Minneapolis, MN). The purity and viability of eosinophils were determined by light microscopic examination of cytocentrifugation preparations (Shandon, Pittsburgh, PA) stained by Diff-Quik (American Scientific Products, McGraw Park, IL) and by trypan blue exclusion, respectively. The purities of eosinophils after MACS beads selection and after FACS were consistently about 50 and 99%, respectively.

*Western blot analysis for MAP kinase phosphorylation.* Chymase-induced activation of MAP kinases was examined by Western blotting. Eosinophils were suspended at $1 \times 10^6$ cells/ml in RPMI1640 containing 1% bovine serum albumin, 25 mM Hepes, and 7.5% $NaHCO_3$ and stimulated by incubating with chymase at 37 °C. Following the stimulation, the cells were resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_3VO_4$, 25 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 1 μg/ml leupeptin), and the lysates were centrifuged to remove insoluble materials. After the supernatants were taken and boiled for 3 min in 2× Laemmli buffer, the proteins were separated on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (4/20 multigel, Daiichi Pure Chemicals, Tokyo, Japan), and transferred to nitrocellulose membrane. The membrane was then incubated in blocking buffer containing 50% Block Ace (Snow Brand Milk Products, Tokyo, Japan) in TBS-T (20 mM Tris, pH 7.2, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature. For detection of MAP kinases and their phosphorylated forms, the membrane was incubated at 4 °C overnight with primary antibody (anti-p44/42 polyclonal antibody, anti-phospho-p44/42 polyclonal antibody, anti-p38 polyclonal antibody, anti-phospho-p38 polyclonal antibody, anti-JNK polyclonal antibody, or anti-phospho-JNK polyclonal antibody; Cell Signaling Technology, Beverly, MA). The membrane was washed with TBS-T and subsequently incubated for 1 h at room temperature with horseradish peroxidase-linked goat anti-rabbit IgG antibody (Cell Signaling Technology) at 1:2000 dilutions. Immunoreactivity was detected by LumiGLO (Cell Signaling Technology).

*Chemotaxis assay.* Chemotaxis assay was carried out using a 48-well microchemotaxis chamber, in which the upper and the lower compartments were separated by a polycarbonate filter with a pore diameter of 5 μM (Neuroprobe, Cabin John, MD). Fifty microliters of cell suspension ($4 \times 10^6$ cells/ml) was placed in the upper chamber and aliquots of either human chymase or mMCP-4 were added in the lower chamber. The chamber was incubated for 1 h at 37 °C in an atmosphere of 5% $CO_2$. After the filter was removed, the cells on the filter were fixed and stained with Hemacolor (Merck Diagnostics, Darmstadt, Germany). The migrated cells adhered to distal part of the filter were counted in three high power fields for each well. To examine the effect of inhibitors in this assay, the cells were preincubated with the inhibitors for 30 min at 37 °C and placed in the lower chamber.

*Statistical analysis.* Data are presented as means ± SD. The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t* test using Statview (SAS Institute), respectively. The *p* value of less than 0.05 was considered significant.

## Results

### *Chymase-induced chemotaxis of human eosinophilic leukemia EoL-1 cells*

To study the intracellular events induced by chymase in eosinophils, we investigated first the effect of human chymase on human leukemia EoL-1 cells. In the initial trail, it was shown that the migration of EoL-1 cells induced by human chymase is little, if any (data not shown). Thus, the cells were subjected to treatment with butyric acid, which is known to induce differentiation of EoL-1 into eosinophil-like cells [19]. As shown in Fig. 1, incubation of EoL-1 cells with butyric acid increased the content of eosinophil-derived neurotoxin (Fig. 1A), the marker for mature eosinophils, in a time-dependent manner. Human chymase significantly stimulated the chemotactic activity of EoL-1 treated with butyric acid for 12 days (Fig. 1B), suggesting that the butyric acid-treated EoL-1 cells possess the machinery required for the chymase-induced cell migration. The concentration of human chymase to stimulate the migration of the