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European Journal of Pharmacology 538 (2006) 175–181
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Mast cell chymase induces expression of chemokines for neutrophils in eosinophilic EoL-1 cells and mouse peritonitis eosinophils

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Received 25 November 2005; received in revised form 22 March 2006; accepted 24 March 2006
Available online 9 May 2006

Abstract

Human chymase induced release of interleukin-8 (IL-8) in human EoL-1 cells that had been differentiated into eosinophil-like cells with butyric acid. The chymase-induced IL-8 production was specific in that other cytokines/chemokines examined were not induced. Human chymase also increased mRNA for IL-8 in the differentiated EoL-1 cells, showing involvement of mRNA synthesis. The chymase-induced IL-8 release was inhibited by pertussis toxin as well as U0126 (an inhibitor for extracellular signal-regulated kinase pathway) and SB203580 (p38 inhibitor), suggesting that the chymase-induced IL-8 production is mediated by G protein-coupled receptor and mitogen-activated protein kinases. Mouse mast cell protease-4 (mMCP-4), a mouse chymase, induced macrophage-inflammatory protein-2 (MIP-2), a mouse homologue for IL-8, in mouse eosinophils in vitro. Intradermal injection of mMCP-4 not only induced skin edema but increased MIP-2 content and neutrophil number at the injection site. Taken together, our findings demonstrate that mast cell chymase may contribute to the interaction between eosinophils and neutrophils by inducing IL-8/MIP-2 in eosinophils at allergic inflamed sites.

Keywords: Chymase; Mast cell; IL-8/MIP-2; Eosinophil; Neutrophil

1. Introduction

Chymase is a chymotrypsin-like serine protease exclusively stored in mast cells and secreted by the cells upon degranulation (Welle, 1997). Chymase cleaves a variety of physiological substances including metalloproteases (Fang et al., 1997), procollagen (Kofford et al., 1997), precursor of interleukin-1β (IL-1β) (Mizutani et al., 1991) and stem cell factor (Longley et al., 1997), while its precise role is not clear. Recently, our data and others have shown that chymase stimulates migration of inflammatory cells including eosinophils in vitro (Tani et al., 2000; Watanabe et al., 2002) as well as in vivo (He and Walls, 1998a; Tomimori et al., 2002), showing that chymase functions as a chemoattractant. The mechanism of the chymase-induced cell migration is not fully elucidated, but it may be mediated by a certain receptor like protease-activated receptor (PAR), as chymase increases intracellular Ca^{2+} concentration (Saito et al., 2003), which is inhibited by pertussis toxin, the inhibitor of G protein-coupled receptor such as PARs (Cocks and Moffatt, 2000).

Eosinophil accumulation in blood and tissues is a hallmark feature of a number of disorders, including allergic diseases (e.g., asthma and atopic dermatitis) and parasite infection (Giembycz and Lindsay, 1999). Eosinophils have been thought to participate in the pathophysiology of such diseases largely by the release of preformed basic protein mediators such as eosinophil peroxidase and major basic protein, which are not only cytotoxic to parasites but damaging to surrounding tissues (Giembycz and Lindsay, 1999). However, recent studies have shown that eosinophils also produce numerous cytokines in response to various stimuli. For example, interferon-γ induces expression of IL-3 mRNA in eosinophils from the patients with allergic diseases (Fujisawa et al., 1994). Th2-like cytokines, IL-4 and granulocyte-macrophage colony-stimulating factor (CSF), induce expression of Th1 cytokine IL-12 in eosinophils from atopic patients, showing that eosinophils may promote a switch from Th2 to Th1 immune responses in the patients (Grewe et al., 1998). In addition, normal eosinophils incubated with immobilized immunoglobulin (Ig) or tumor necrosis factor-α (TNF-α) release IL-8 (Nakajima et al., 1996), a potent neutrophil chemotactic factor (Mukaida, 2003). Interestingly, moreover, IL-8 is also produced in eosinophils by stimulating with tryptase which is, like chymase, released from

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doi:10.1016/j.ejphar.2006.03.053

mast cells (Temkin et al., 2002). These findings demonstrate that eosinophils have regulatory roles in immune and inflammatory reactions in addition to their effector function to parasites and tissues.

In the present study, we examined the ability of mast cell chymase to induce cytokine/chemokine synthesis using human eosinophilic cell line EoL-1 as well as eosinophils from mouse peritonitis, and the results showed for the first time that chymase may stimulate eosinophils to produce chemokines for neutrophils, IL-8 and macrophage-inflammatory protein-2 (MIP-2), in human and mouse eosinophils, respectively.

2. Materials and methods

2.1. Reagents

Human chymase was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant mouse mast cell protease-4 (mMCP-4) was prepared as described (Watanabe et al., 2002). Chymase inhibitor SUN C8257 (3-[(3-amino-4-carboxy)phenylsulfonyl]-7-chloroquinazorine 2,4(1*H*,3*H*)-dione) have been synthesized as described previously (Fukami et al., 2000). Pertussis toxin was purchased from BIOMOL, Inc. (Plymouth Meeting, PA). Mitogen-activated protein (MAP) kinase inhibitors, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) and SB203580 (4-[5-(4-fluoro-phenyl)-2-(4-methanesulfinyl-phenyl)-3*H*-imidazol-4-yl]-pyridine) were purchased from CN Biosciences, Inc. (San Diego, CA). Actinomycin D and cycloheximide were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

2.2. Mice

BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). All animal experiments were approved by Institutional Review Board for Animal Studies of Daiichi Asubio Pharma Co. Limited, Biomedical Research Laboratories.

2.3. Human eosinophilic cell line EoL-1

A human eosinophilic leukemia cell line EoL-1 (Saito et al., 1985) 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 previously (Saito et al., 1993).

2.4. Isolation of mouse eosinophils

Mouse eosinophils were isolated from the peritoneal fluid of the mice with allergen-induced peritonitis (Terakawa et al., 2005). Briefly, BALB/c mice were immunized by subcutaneous injection with ragweed extract (1/1000 dilution, Torii Pharmaceutical Co., 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 were purified by negative selection using Thy1.2 and B220 MACS beads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) according to the manufacturer's instruction and then purified by fluorescence-activated cell sorting (FACS) with phycoerythrin-conjugated anti-CCR3 monoclonal antibodies (R&D Systems Inc., 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 purity of eosinophils was consistently about 99%.

2.5. Detection and measurement of cytokines/chemokines

Comprehensive analysis of cytokine/chemokine production by the cells was examined using a solid phase multiplexed protein assay kit (Cartesian Array Human Cytokine Set 1, Biosource Inc, Camarillo, CA). The concentration of IL-8, MIP-2 and KC were determined using enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (Biosource, Inc, Camarillo, CA), MIP-2 and KC (R&D Systems, Inc).

2.6. Measurement of mRNA for IL-8

Total RNA was extracted with Isogen (Nippon Gene, Osaka, Japan) and the first-strand cDNA was synthesized using SuperScript II RNase H-reverse transcriptase (RT) (Gibco-BRL Life Technologies, Eggenstein, Germany) and Oligo-dT25 (Gibco-BRL Life Technologies). The generated cDNA was amplified using 1.25 U of Taq DNA polymerase, dNTP mixture and primers for IL-8, IL-6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The sequences of the primers are as follows.

IL-8: 5′-AAG ACA TAC TCC AAA CCT TTC CAC C-3′ for the 5′ primer, 5′-ACA ACC CTC TGC ACC CAG TT-3′ for the 3′ primer, spanning a fragment of 155 bp
IL-6: 5′-TCT CAG CCC TGA GAA AGG AGA C-3′ for the 5′ primer, and 5′-GAA GAG CCC TCA GGC TGG ACT G-3′ for the 3′ primer, spanning a fragment of 438 bp
GAPDH: 5′-ACC ACA GTC CAT GCC ATC AC-3′ for the 5′ primer, and 5′-TCC ACC ACC CTG TTG CTG TA-3′ for the 3′ primer, spanning a fragment of 452 bp

Polymerase chain reaction (PCR) amplification was performed using a thermal cycler (GeneAmp PCR System 9700, PE Biosystems) under the following conditions: one initial denaturation cycle for 1 min at 94 °C; 35 amplification cycles for 1 min at 94 °C, 1 min at 62 °C and 1 min at 72 °C; and a final extension phase consisting of one cycle of 10 min at 72 °C. The PCR products were visualized on a 1.5% agarose gel (Gibco-BRL Life Technologies) containing 0.05 mg/ml ethidium bromide (Sigma-Aldrich Japan).

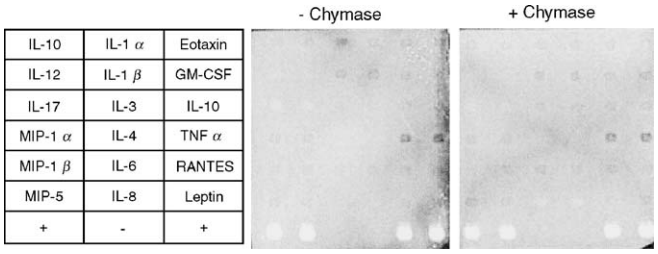

Fig. 1. Effect of human chymase on release of various cytokines/chemokines from human eosinophilic EoL-1 cells. Human eosinophilic leukemia EoL-1 cells cultured in the presence of 0.5 mM of butyric acid for 12 days were incubated with 4 μM of human chymase for 24 h and a panel of cytokines/chemokines in the culture supernatant was detected as described in Materials and methods. Detection of cytokines/chemokines indicated in the left was performed in duplicate. The photo is the representative results of the two separate experiments.

2.7. Ear edema induced by mMCP-4 injection

mMCP-4 was dissolved in saline and 10 μl of the solution was injected intradermally (2.0 μg/site) to the right ear of BALB/c mice. At 18 h following the injection, the ear samples containing the injected site were taken using a punch (a diameter of 6 mm, Kai industries, Gifu, Japan) and the edematous reaction was evaluated by weighing the ear biopsy. The edema was expressed as the difference in the weight of the ear punch biopsy between the right and the left ears of the same mouse. In control group, the same volume of saline was injected to the mice. The ear specimens were also used for measurement of MIP-2 content and histological analysis. For MIP-2 measurement, the ear samples were minced and homogenized in 20 mM Tris–HCl buffer pH 7.5. The homogenate was subsequently centrifuged at 10,000×*g* for 30 min and the supernatant was used for the assay using ELISA.

2.8. Histological analysis

Ear samples were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxylin and eosin. The number of dermal infiltrated neutrophils was determined by counting the stained cells in a high-power field (×400) for each section.

2.9. Statistical analysis

Data are presented as mean±S.D. or mean±S.E.M. The statistical analysis was performed with Dunnett's multiple comparison test or Student's *t*-test using Statview (SAS Institute Inc.), respectively. The *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Effect of human chymase on cytokine/chemokine production in human leukemia EoL-1 cells

We have recently shown that incubation of EoL-1 cells with butyric acid increases content of eosinophil-derived neurotoxin in the cells, and that human chymase significantly stimulates the chemotactic activity of EoL-1 treated with butyric acid (Terakawa et al., 2005). These data show that the butyric acid-treated EoL-1 cells possess eosinophilic properties as well as the machinery necessary for the chymase-induced changes of cellular function. As shown in Fig. 1, butyric acid-treated EoL-1 cells spontaneously released IL-17, but the release was not increased by treatment with human chymase. On the other hand, IL-8, a chemokine for neutrophils, was released from the cells following the chymase treatment, while it was not detected in the culture supernatant of the untreated cells (Fig. 1). The other cytokines including IL-1α, IL-1β, IL-3, IL-4, IL-6, TNF-α and eotaxin were not released even after the chymase treatment.

3.2. Effect of human chymase on IL-8 release in human leukemia EoL-1 cells

To examine the kinetics and concentration dependency of the chymase-induced IL-8 release by EoL-1 cells, IL-8 content was quantified by ELISA. As shown in Fig. 2A, the chymase-induced IL-8 release from butyric acid-treated EoL-1 cells was time- and

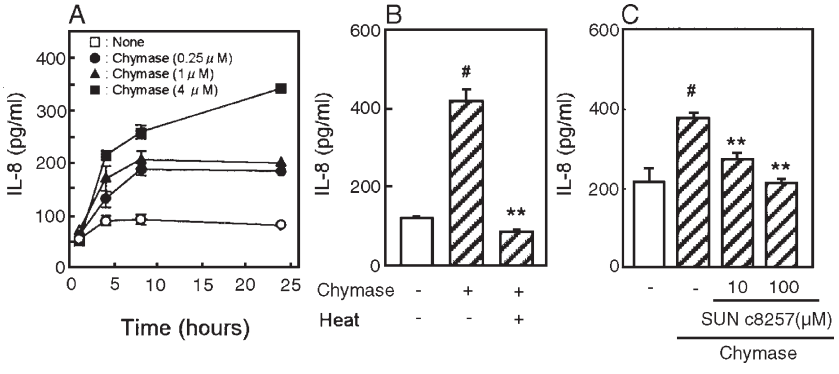

Fig. 2. Effect of human chymase on IL-8 release from human eosinophilic leukemia EoL-1 cells. EoL-1 cells cultured in the presence of 0.5 mM of butyric acid for 12 days were incubated with various concentrations (A) or 1.0 μM (B, C) of human chymase for the indicated times (A) or 24 h (B, C), and IL-8 was measured by ELISA. (A) Time- and concentration-dependency of the chymase-induced IL-8 release from EoL-1 cells. (B) Effect of heat-treated human chymase on IL-8 release from EoL-1 cells; open bar, without chymase, hatched bar, 1 μM of human chymase or heat-treated human chymase. (C) Effect of chymase inhibitor SUN C8257 on chymase-induced IL-8 release from EoL-1 cells; open bar, without human chymase; hatched bars, 1 μM of human chymase. Data are mean±S.D. ($n=3$). $^{\#}P<0.01$ compared with the untreated control (without human chymase); $^{**}P<0.01$ compared with the control (treated with intact human chymase).

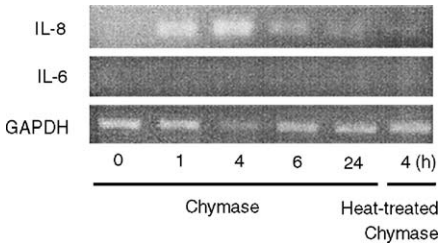

Fig. 3. Effect of human chymase on expression of mRNA for IL-8 in human eosinophilic leukemia EoL-1 cells. EoL-1 cells cultured in the presence of 0.5 mM of butyric acid for 12 days were incubated with 1.0 μM of intact human chymase or heat-treated human chymase, and mRNAs for IL-8, IL-6 and GAPDH were measured by RT-PCR as described in Materials and methods. The photo is the representative results of the two separate experiments.

concentration-dependent; the IL-8 level started to increase by 4 h following the chymase treatment and reached to the maximal level at around 8 h at less than 1 μM of chymase, while the level continued to increase at least for 24 h when treated with 4 μM of chymase. Heat-inactivated human chymase failed to induce IL-8 release from butyric acid-treated EoL-1 cells (Fig. 2B), and the chymase-induced IL-8 release from EoL-1 cells was inhibited by a chymase inhibitor SUN C8257 (Fig. 2C). These results indicate that the ability of chymase to induce IL-8 production depends on its enzymatic activity of chymase.

3.3. Involvement of mRNA synthesis in chymase-induced IL-8 release in EoL-1 cells

Next, we examined the mechanism of the IL-8 release induced by chymase in EoL-1 cells using RT-PCR and inhibitors for protein and RNA synthesis. As shown in Fig. 3, human chymase also induced expression of mRNA for IL-8 in butyric acid-treated EoL-1 cells. The chymase-induced increase in the level of IL-8 mRNA was time-dependent with a maximal level observed at 1–4 h, showing that the induction of mRNA for IL-8 precedes that of IL-8 protein. Again, the effect of chymase was specific for IL-8, since expression of IL-6 mRNA was not increased by the chymase treatment. In addition, heat-inactivated chymase did not increase IL-8 mRNA (Fig. 3). Moreover, addition of actinomycin D or cycloheximide significantly blocked the chymase-induced IL-8 release from EoL-1 cells (Fig. 4). These data suggest that the chymase-induced IL-8 release from EoL-1 requires the de novo synthesis of protein and mRNA for IL-8.

3.4. Effect of pertussis toxin and MAP kinase inhibitors on chymase-induced IL-8 production in EoL-1 cells

We have recently shown that the ability of chymase to induce intracellular Ca^{2+} influx is inhibited by pertussis toxin, the inhibitor of G protein-coupled receptor (Saito et al., 2003), and that chymase induces phosphorylation of extracellular signal-regulated kinase (ERK) and p38 but not that of c-Jun N-terminal protein kinase (JNK) in EoL-1 and mouse eosinophils (Terakawa et al., 2005). Thus, we next examined whether G protein-coupled receptor, ERK and p38 are also involved in the chymase-induced IL-8 production. As shown in Fig. 5A, the chymase-induced IL-8 release from EoL-1 cells was inhibited by pertussis toxin in a concentration-dependent manner. In addition, the chymase-induced IL-8 production was also reduced by the treatment with U0126 (inhibitor of ERK pathway) or SB203580 (p38 inhibitor). These data suggest that the chymase-induced IL-8 production is mediated by G protein-coupled receptor as well as ERK and p38 signal transduction pathways.

3.5. Effect of chymase on chemokine production in mice

It is known that murine functional homologues for human IL-8 are MIP-2 and KC (Cochran et al., 1983; Tekamp-Olson et al., 1990). To investigate whether chymase also functions in mice as a stimulator of chemokine production, we examined the effect of mouse chymase mMCP-4 on production of MIP-2 and KC using mouse eosinophils in vitro. mMCP-4 treatment concentration-dependently induced production of MIP-2 (Fig. 6A), whereas KC was not at all detected after the same treatment (data not shown). In in vivo experiment, an intradermal injection of mMCP-4 to mouse ear significantly induced skin edema at the injection site (Fig. 6B). Moreover, the mMCP-4 injection significantly increased content of MIP-2 (Fig. 6C) as well as the number of neutrophils (Fig. 6D). These data demonstrate that the ability of chymase to induce chemokine for neutrophil is conserved between human and mouse and therefore may have a physiological importance.

4. Discussion

The data in the present study showed that human mast cell chymase induces release of IL-8 from human eosinophilic cell line EoL-1 in a time- and concentration-dependent manner (Fig. 2). The release of IL-8 was mediated by expression of mRNA for IL-8 in the cells (Figs. 3 and 4). The chymase-induced cytokine release by EoL-1 was specific for IL-8 in that other chemokines/cytokines such as IL-3, IL-4 and IL-6 were

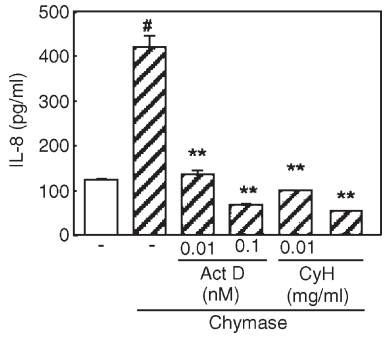

Fig. 4. Effect of actinomycin D and cycloheximide on chymase-induced IL-8 production in human eosinophilic leukemia EoL-1 cells. EoL-1 cells cultured in the presence of 0.5 mM of butyric acid for 12 days were incubated with 1.0 μM of human chymase for 24 h in the presence of actinomycin D (Act D) (A) or cycloheximide (CyH) (B). IL-8 was measured by ELISA as described in Materials and methods. Open bar, without chymase; hatched bars, 1 μM of human chymase. Data are mean±S.D. ($n=3$). $^{\#}P<0.01$ compared with the untreated control (without human chymase); $^{**}P<0.01$ compared with the control (treated with human chymase alone).

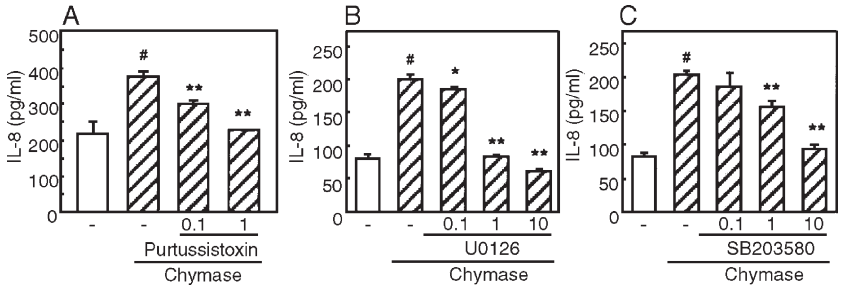

Fig. 5. Effect of pertussis toxin and MAP kinase inhibitors on chymase-induced IL-8 release from human eosinophilic leukemia EoL-1 cells. EoL-1 cells cultured in the presence of 0.5 mM of butyric acid for 12 days were incubated with 1.0 μM of human chymase for 24 h in the presence of pertussis toxin (A), U0126 (B) or SB203580 (C), and IL-8 in the culture supernatant was measured by ELISA. Data are mean±S.D. (n=3). $^{\#}P<0.01$ compared with the untreated control (without human chymase); $^{*}P<0.05$; $^{**}P<0.01$ compared with the control (treated with human chymase alone).

not released by the treatment with chymase (Fig. 1). Similarly, mouse chymase mMCP-4 induced release of MIP-2, a mouse homologue of IL-8, in vitro and in vivo (Fig. 6). These findings suggest that chymase secreted by mast cells play a role in promoting interaction between eosinophils and neutrophils, as IL-8 and MIP-2 are the major chemokine for neutrophils in human and mouse, respectively.

Injection of chymase into the mouse peritoneum is known to increase eosinophils as well as neutrophils in the peritoneal cavity (He and Walls, 1998a). This finding was supported by our data that intradermal injection of mMCP-4 increased the number of neutrophils at the injected site (Fig. 6). Chymase stimulates migration of both eosinophils and neutrophils in vitro (Tani et al., 2000), indicating that chymase-induced neutrophil accumulation in vivo is, at least in part, due to the chemotactic activity of chymase to neutrophils. Therefore, the chymase-induced neutrophil accumulation may be mediated by both direct and IL-8/MIP-2-mediated indirect mechanisms.

In the in vivo study of the present study, there are no clear data indicating the causal relation between edema, MIP-2 and neutrophil infiltration, although the extents of the increase in these parameters seem to be similar (Fig. 6). It is known, on the other hand, that chymase has an ability to increase vascular permeability (He and Walls, 1998b), suggesting that the chymase-induced edema may be partly mediated by this activity. The experiment using neutralizing antibody to MIP-2 could make clear the importance of MIP-2 in neutrophil accumulation and edema induced by mMCP-4.

The chymase-induced IL-8 release from EoL-1 was significantly blocked by pertussis toxin, MAP kinase kinase (MEK) inhibitor U0126 and p38 inhibitor SB203580 (Fig. 5), showing the involvement of G protein-coupled receptor as well as ERK and p38 MAP kinases in the chymase-induced IL-8 production in eosinophils. These data are consistent with our recent finding that chymase induces rapid phosphorylation of ERK and p38 but not JNK in EoL-1 cells as well as mouse eosinophils (Terakawa et al., 2005). Recently, Temkin et al. have reported that mast cell tryptase induces IL-8 release from human eosinophils, which is inhibited by any of the inhibitors of ERK pathway, p38 and JNK (Temkin et al., 2002). Their findings and ours suggest that distinct mechanisms mediate the chymase- and tryptase-induced IL-8 production in eosinophils, although both enzymes are stored in mast cell granules and their receptor are G protein-coupled receptors. Supporting this notion is that the spectrum of the cytokine/chemokine production induced by chymase and tryptase are different, e.g., tryptase induces IL-6 release (Temkin et al., 2002), whereas chymase does not (Fig. 1). In this respect, it should be noted that the receptor for tryptase is thought to be PAR2 (Molino et al., 1997), but that for chymase has not yet been identified.

The mast cells containing chymase are the connective-tissue type but not the mucosal type, in contrast to tryptase that is stored both types of the cells (Welle, 1997). Thus, the chymase-mediated IL-8/MIP-2 production would be associated with the

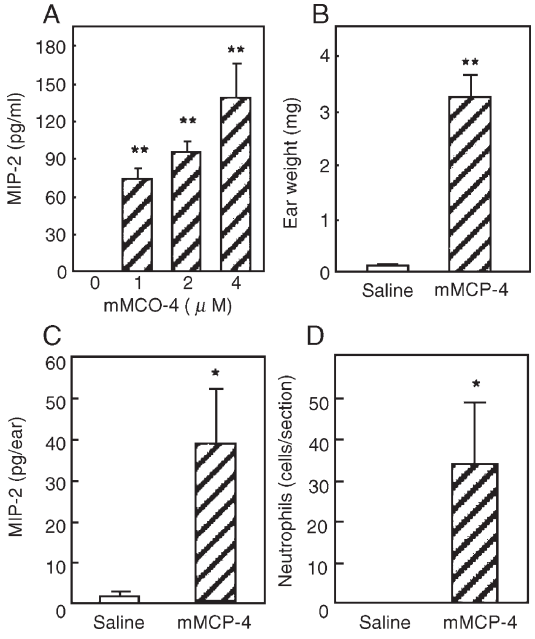

Fig. 6. Ability of mMCP-4 to induce MIP-2 production, edema formation and neutrophil accumulation. (A) Mouse eosinophils were purified from peritonitis mouse and incubated with various concentrations of mMCP-4 for 24 h. The contents of MIP-2 in the culture supernatant were measured by ELISA. (B–D) mMCP-4 in saline was intradermally injected into the mouse ear at 2 μg/site and the edematous reaction (B) was evaluated by weighing the ear biopsy. The content of MIP-2 (C) in the ear specimens was measured as described in Materials and methods. The number of infiltrating neutrophils (cells/section) in dermis (D) was counted in high-power fields (×400) using ear sections. For the ear of control mice, saline was injected instead of mMCP-4. Data are mean±S.D. (n=3) (A) or mean±S.E.M. (n=6 or 15) (B–D). $^{*}P<0.05$; $^{**}P<0.01$ compared with the control.

role of neutrophils in connective tissues such as the skin. In fact, as mentioned above, intradermal injection of mMCP-4 induced neutrophil infiltration in the skin. IL-8 content is known to be significantly greater at the antigen challenge sites during the IgE-mediated late-phase reaction in human (Wershil et al., 1991). In addition, it has been reported that recruitment of neutrophils during IgE-dependent cutaneous late phase reaction in mice is mast cell dependent, and that anti-TNF-α antibody partially inhibits the neutrophil accumulation (Zweiman et al., 1998). It is thus possible that mast cell chymase may contribute to the neutrophil accumulation in the late-phase skin reaction by stimulating IL-8/MIP-2 production in eosinophils.

It is well known that there is a cross-talk between mast cells and eosinophils in allergic reaction. For example, mast cell-derived TNF-α enhances eosinophil survival by inducing production of autocrine survival cytokine GM-CSF in eosinophils (Levi-Schaffer et al., 1998). Histamine released from mast cells is known to stimulate chemotaxis of eosinophil through H4 receptor on eosinophils (Buckland et al., 2003; Ling et al., 2004). Conversely, eosinophils have been shown to activate IgE-challenged and immunologically desensitized mast cells, which is partly mediated by major basic protein (Piliponsky et al., 2001). The data in the present study have shown that mast cell chymase induces release of neutrophil chemoattractant IL-8/MIP-2 by eosinophils. Our findings suggest that neutrophil is an additional member involved in the cell–cell interaction in allergic inflammation.

Acknowledgements

We thank Dr. Toshihiro Nakanishi for his critical reading and suggestions to the manuscript.

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