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Mast Cell β-Tryptase Selectively Cleaves Eotaxin and RANTES and Abrogates Their Eosinophil Chemotactic Activities1

Linhua Pang,2 Mei Nie, Lisa Corbett, Amy Sutcliffe, and Alan J. Knox

Recent studies have shown that a lack of eosinophils in asthmatic airway smooth muscle (ASM) bundles in contrast to the large number of mast cells is a key feature of asthma. We hypothesized that this is caused by β-tryptase, the predominant mast cell-specific protease, abrogating the eosinophil chemotactic activities of ASM cell-derived eosinophil chemotactants such as eotaxin and RANTES. We studied the effect of β-tryptase on the immunoreactivities of human ASM cell-derived and recombinant eotaxin and other recombinant chemokines that are known to be produced by human ASM cells. We report in this study that purified β-tryptase markedly reduced the immunoreactivity of human ASM cell-derived and recombinant eotaxin, but had no effect on eotaxin mRNA expression. The effect was mimicked by recombinant human β-tryptase in the presence of heparin and was reversed by heat inactivation and the protease inhibitor leupeptin, suggesting that the proteolytic activity of tryptase is required. β-tryptase also exerted similar effects on recombinant RANTES, but not on the other chemokines and cytokines that were screened. Furthermore, a chemotaxis assay revealed that recombinant eotaxin and RANTES induced eosinophil migration concentration-dependently, which was abrogated by pretreatment of these chemokines with β-tryptase. Another mast cell protease, chymase, also markedly reduced the immunoreactivity of eosinotaxin, but had no effect on RANTES and other chemokines and did not affect the influence of β-tryptase on RANTES. These findings suggest that mast cell β-tryptase selectively cleaves ASM-derived eotaxin and RANTES and abrogates their chemotactic activities, thus providing an explanation for the eosinophil paucity in asthmatic ASM bundles.

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Tryptase is also an extremely potent mitogen for ASM cells (13), and in contrast, chymase profoundly inhibits mitogen-induced ASM cell proliferation (14). These observations strongly suggest that there are complex interactions between ASM cells and mast cells.

Inflammatory cells are recruited to local tissues by a complex network of chemokines and other chemotactic factors. Eotaxin is a potent eosinophil chemoattractant, acting selectively through the CCR3. RANTES is also a potent chemoattractant for eosinophils (15), memory T cells, and monocytes (16). Proteolysis is one means by which chemokine activity can be modulated. For example, RANTES, MCP-2, eotaxin, and IFN-γ-inducible protein 10 are substrates of dipetidyl peptidase IV (CD26), a leukocyte activation marker and a serine protease (17), and truncated RANTES molecule exerts different activities from those of RANTES because of its altered receptor specificity (17). Eotaxin cleaved at the N terminus by CD26 also shows a loss of its chemotactic potency (18). Furthermore, proteases produced by the adult hookworm Necator americanus cleave eotaxin, but not IL-8 or eotaxin-2, resulting in a loss of its immunoreactivity as well as its chemotactic activity (19).

Human ASM cells are a rich source of eotaxin (6, 20) and RANTES (10). However, despite the facts that strong signals of eotaxin mRNA and immunoreactivity are observed in vivo in smooth muscle in asthmatic airways (20) and that RANTES mRNA is elevated in airways of patients with asthma (21), there is an eosinophil paucity in asthmatic ASM bundles in contrast to the large number of mast cells (1). Because mast cells present in asthmatic airways are in a chronically activated secretory state (22), we hypothesize that tryptase secreted from microlocalized mast cells may reduce the chemotactic capability of ASM cell-derived eosinophil chemoattractants, eotaxin and RANTES in particular, either through inhibition of their expression or through cleavage of preformed chemokines. In this study we showed that among the chemokines and cytokines screened, eotaxin and RANTES were selectively cleaved by tryptase, which resulted in the loss of their eosinophil chemotactic potency. These findings thus suggest that the selective cleavage of eotaxin and RANTES by mast cell tryptase contributes to the eosinophil paucity seen in asthmatic ASM bundles.

Materials and Methods

Materials

Purified human lung tryptase was obtained as a liquid containing 50 mM NaOAc, 1 M NaCl, and 0.05 mM heparin (pH 5.0) with 0.01% NaN₃ from Europa Bioproducts (sp. act., 12,870 mU/mg protein using 4-N benzyl-D,L-arginine-pNA as substrate); purified human skin chymase was obtained from Merck; the RNeasy minikit was purchased from Qiagen; Moleotaxin and eotaxin-2, and human eosinophil enrichment column kit were purchased from R&D Systems. Briefly, blood was mixed with RBC gradient solution and left for 30–45 min to allow RBC sedimentation. The top plasma layer of cells was added on top of the polymorphonuclear cell (PMN) separation medium. After centrifugation at 500 × g for 30 min, the cell pellet containing PMNs was collected. The remaining RBCs were lysed by hypotonic wash. Up to 2 × 10⁷ PMNs were incubated with 1 ml of the Ab mixture for 20 min, washed with column buffer, then applied to the eosinophil selection column and incubated for 10 min. The eosinophils were finally eluted with column buffer (>97% purity) and resuspended in RPMI 1640 medium containing 1% serum. This protocol was approved by Nottingham University Medical School research ethics committee, and all human participants gave written informed consent.

Chemotaxis assay

The migration of eosinophils in response to eotaxin and RANTES in vitro was assessed using 5-μm pore size polycarbonate membrane Transwell inserts in 24-well culture plates (Corning). Medium alone (0.6 ml) or medium containing recombinant chemokines with or without neutralizing Ab or tryptase was added into the wells. Purified eosinophils (1 × 10⁷) in 0.1 ml of RPMI 1640 containing 1% serum were then placed into the inserts, which were then placed into the wells. The plates were incubated at 37°C and 5% CO₂ for 90 min. Eosinophils that migrated through the filter into the lower chamber were collected, resuspended, and counted. Chemotaxis was expressed as a migration index (the ratio of migrated cell numbers from experimental groups divided by those from control groups) (25, 26).

Statistical analysis

Data were expressed as the mean ± SE. Statistical analysis was performed with GraphPad PRISM (version 4). Unpaired two-tailed Student’s t test was used to determine the significant differences between the means; p < 0.05 was accepted as statistically significant.

Results

Tryptase reduces the immunoreactivity of human ASM cell-derived eotaxin without affecting its mRNA expression

To examine the effect of tryptase on ASM cell-derived eotaxin immunoreactivity, cells were treated with or without tryptase for 24, 48, and 72 h. Eotaxin was released from ASM cells constitutively, but treatment with tryptase at all concentrations markedly reduced eotaxin levels in the medium (Fig. 1A). To test the effect of tryptase on TNF-α-induced immunoreactive eotaxin, cells were
mRNA expression, cells were pretreated with or without tryptase.

reactivity from ASM cells was due to its inhibition of eotaxin
quire on an intact catalytic site in this process.
pletely reversed the effect of tryptase (Fig. 1B), suggesting a re-
Heat inactivation of tryptase (30 mU/ml) completely abolished the effect of tryptase.

FIGURE 1. Effect of tryptase on human ASM cell-derived eotaxin immunoreactivity and mRNA expression. A, ASM cells in 24-well plates were treated with increasing concentrations of tryptase for the times indicated. B, ASM cells were pretreated with or without tryptase for 30 min before incubation with TNF-α for 24 h. Eotaxin levels in the medium were measured by ELISA. Each point represents the mean ± SE of two experiments performed in triplicate. C, ASM cells were pretreated with or without tryptase for 30 min before incubation with TNF-α for the times indicated. mRNA levels of eotaxin and GAPDH were measured by RT-PCR. Results are representative of three independent experiments with similar results. D, ASM cells in 90-mm dishes were treated with TNF-α for 24 h. Cell-free medium was collected, and aliquots were incubated at 37°C with or without increasing concentrations of tryptase or heat-inactivated tryptase for 16 h. Eotaxin levels in the medium were measured by ELISA. Each point represents the mean ± SE of two experiments performed in triplicate.

pretreated with or without tryptase for 30 min, then incubated in the presence or the absence of TNF-α for 24 h. TNF-α markedly increased eotaxin levels in the medium compared with control cells, but the increase was concentration-dependently abolished by tryptase (Fig. 1B). Heat inactivation of tryptase (30 mU/ml) completely reversed the effect of tryptase (Fig. 1B), suggesting a requirement for an intact catalytic site in this process.

To explore whether the effect of tryptase on eotaxin immunoreactivity from ASM cells was due to its inhibition of eotaxin mRNA expression, cells were pretreated with or without tryptase for 30 min before incubation with TNF-α. Eotaxin mRNA was constitutively expressed in ASM cells, and expression was markedly enhanced by TNF-α after 2-h treatment and was maintained up to 20 h. However, tryptase had no effect on TNF-α-induced eotaxin mRNA expression at any time point (Fig. 1C), suggesting that tryptase-induced loss of immunoreactivity of ASM cell-derived eotaxin is likely to be post-transcriptional, involving its proteolytic activity.

To confirm this, the effect of tryptase on the immunoreactivity of preformed eotaxin was assessed. Medium from TNF-α-treated ASM cells (10 ng/ml, 24 h) was incubated at 37°C for 16 h in the presence or the absence of tryptase, and the remaining immunoreactive eotaxin was measured by ELISA. As shown in Fig. 1D, incubation of the medium in the absence of tryptase resulted in a small loss of eotaxin measurable by ELISA compared with the control (without incubation). However, incubation in the presence of tryptase led to a concentration-dependent reduction and eventual abolishment of immunoreactive eotaxin, which was reversed by heat inactivation of tryptase, suggesting that proteolysis was responsible for the loss of immunoreactivity of preformed eotaxin.

Tryptase reduces the immunoreactivity of recombinant eotaxin

To examine the effect of tryptase on the immunoreactivity of recombinant eotaxin, recombinant human eotaxin (500 pg/ml) was incubated with increasing concentrations of tryptase at 37°C for 16 h. As shown in Fig. 2A, a small loss of immunoreactive eotaxin was observed after incubation in the absence of tryptase, whereas tryptase reduced immunoreactive eotaxin in a concentration-dependent manner. A marked reduction was observed at a concentration as low as 0.007 mU/ml, and complete abolishment was achieved with 7.5 mU/ml tryptase. To assess the effect of tryptase (7.5 mU/ml), recombinant human eotaxin at increasing concentrations was incubated at 37°C for 16 h in the presence or the absence of tryptase. As shown in Fig. 2B, tryptase (7.5 mU/ml) abolished eotaxin immunoreactivity regardless of its concentration. To explore the time course of the tryptase effect, recombinant human eotaxin (1000 pg/ml) was incubated at 37°C in the presence or the absence of tryptase for up to 15 min. Compared with the small natural loss of eotaxin immunoreactivity in the absence of tryptase (Fig. 2C), a marked loss of eotaxin immunoreactivity occurred immediately after the addition of tryptase to the reaction, and no measurable eotaxin immunoreactivity was detected after 2-min incubation.

To determine whether tryptase could exert a similar proteolytic effect on eotaxin-2, recombinant human eotaxin and eotaxin-2 (both 40 ng) were incubated at 37°C in the presence or the absence of tryptase for up to 30 min, and the remaining immunoreactivities were analyzed by Western blotting. In the absence of tryptase, the immunoreactivities of both eotaxin and eotaxin-2 remained unchanged after incubation (Fig. 2D). However, the immunoreactivity of eotaxin was markedly reduced after incubation with tryptase for 2 min and was undetectable after that, whereas the immunoreactivity of eotaxin-2 remained unchanged for the entire course. Incubation of eotaxin with heat-inactivated tryptase completely reversed the proteolytic effect of tryptase, but did not alter the immunoreactivity of eotaxin-2 (Fig. 2D).

Recombinant human tryptase also reduces eotaxin immunoreactivity, and leupeptin reverses the effect

To test whether rh-tryptase could exert a similar effect on eotaxin immunoreactivity as purified tryptase, recombinant eotaxin (500 pg/ml) was incubated with increasing concentrations of rh-tryptase (0.029–7.5 mU/ml) in the absence or the presence of heparin for
16 h, and the remaining immunoreactive eotaxin was then measured by ELISA. In the absence of heparin, rh-tryptase had no effect on eotaxin immunoreactivity. Heparin alone (29.14 nM) also had no effect. However, in the presence of heparin, rh-tryptase concentration-dependently reduced eotaxin immunoreactivity (Fig. 3A).

Two protease inhibitors, leupeptin and SBTI, were used to explore whether the cleavage of eotaxin by tryptase and rh-tryptase was due to the proteolytic effect of tryptase. Leupeptin inhibits several proteases, including tryptase, and SBTI inhibits proteases other than tryptase (27). As shown in Fig. 3B, both tryptase (0.469 mU/ml) and rh-tryptase (7.5 mU/ml) abolished eotaxin immunoreactivity, and the effect was markedly reversed by preincubation with leupeptin, but not SBTI (both 50 μM). The results also demonstrate that the cleavage of eotaxin is through the proteolytic activity of tryptase.

Tryptase reduces the immunoreactivity of RANTES, but not the other chemokines and cytokines screened

To determine whether tryptase could also cleave other chemokines and cytokines that are known to be produced by human ASM cells, recombinant human RANTES, GM-CSF, MCP-1, IL-8, TGF-β1 and VEGF (all 31.25–1000 pg/ml) were incubated at 37°C in the presence or the absence of tryptase for 16 h, and the remaining immunoreactive chemokines and cytokines were measured by ELISA. In the absence of tryptase, the immunoreactivities of all tested chemokines and cytokines remained almost unaltered after incubation; however, in the presence of tryptase, the immunoreactivity of RANTES was markedly reduced (Fig. 4A), whereas the immunoreactivities of the others were not changed compared with the control (without tryptase; Fig. 4B–F). These results indicate that the cleavage of eotaxin and RANTES by tryptase is selective.

Tryptase abrogates the functional activities of eotaxin and RANTES

Two functional studies were conducted to determine whether the cleavage of eotaxin and RANTES by tryptase could result in consequent loss of their functional activities. As shown in Fig. 5A, TNF-α markedly increased immunoreactive MCP-1 from human...
ASM cells after incubation for 16 h, and the effect was significantly inhibited by recombinant human eotaxin in a concentration-dependent manner. However, the inhibition was completely reversed when eotaxin was preincubated with tryptase at 37°C for 30 min, despite the fact that tryptase on its own had no effect on TNF-α-induced MCP-1 immunoreactivity. Both recombinant human eotaxin and RANTES induced human eosinophil migration in a concentration-dependent manner (Fig. 5, B and C). Tryptase on its own had no effect on eosinophil migration, but pretreatment of eotaxin and RANTES with tryptase at 37°C for 30 min abrogated their eosinophil chemotactic activities (Fig. 5, B and C). The chemotactic effect of eotaxin was also abolished by the anti-eotaxin neutralizing Ab (Fig. 5B). Collectively, these results indicate that tryptase cleavage of eotaxin and RANTES results in the loss of their immunoreactivities as well as their functional activities.

Chymase reduces the immunoreactivity of eotaxin, but not the other chemokines screened

Because the mast cells microlocalized in asthmatic ASM bundles are mainly MC₆C₆ (1), and chymase may exert different or even opposite effects from tryptase (14), the effect of purified chymase, either alone or in combination with tryptase, on the immunoreactivities of recombinant chemokines was studied. Incubation of eotaxin (31.25–1000 pg/ml) with chymase (7.5 mU/ml) at 37°C for 16 h resulted in an almost complete loss of immunoreactive eotaxin, in a similar manner as that of tryptase (7.5 mU/ml) alone and tryptase plus chymase (Fig. 6A). However, chymase had no effect on RANTES immunoreactivity or tryptase-induced loss of immunoreactive RANTES (Fig. 6B). Chymase, either alone or in combination with tryptase, also had no effect on the immunoreactivities of IL-8 (Fig. 6C) and MCP-1 (Fig. 6D). These results indicate that chymase has a similar proteolytic effect on eotaxin as tryptase, but does not cleave the other chemokines screened or alter the cleavage of RANTES by tryptase.

**Discussion**

Mast cell microlocalization and associated eosinophil paucity within the ASM bundle are important determinants of the asthmatic phenotype (1). We have found in this study that mast cell tryptase selectively cleaves eotaxin and RANTES, but not other chemokines and cytokines that human ASM cells are known to produce, and abrogates their eosinophil chemotactic activities. Both eotaxin and RANTES are potent eosinophil chemoattractants and constitute >70% of eosinophil chemotaxis of TNF-α-stimulated ASM cell supernatant (20). The eosinophil paucity seen in asthmatic ASM bundles is therefore probably attributable to the selective cleavage of both chemokines by tryptase released from microlocalized mast cells.

Tryptase has been previously shown to cleave certain extracellular substrates, including vasoactive intestinal peptide and calcitonin gene-related peptide (28), 72-kDa gelatinase, fibronectin (29), and prostromelysin (30). More recently, it has been established that tryptase is a potent growth factor for a number of cell types, including fibroblasts (31), epithelial cells (32), and ASM cells (13). It is of particular importance to note that tryptase can stimulate the release of chemokine IL-8 from epithelial cells (32), endothelial cells (33), and ASM cells (our unpublished observation), suggesting that tryptase may regulate the release of chemokines and subsequently the migration of inflammatory cells. In this study we found that tryptase reduced the release of immunoreactive eotaxin from ASM cells due to the proteolysis of preformed eotaxin, but not the inhibition of its mRNA expression. The loss of eotaxin immunoreactivity was accompanied by the loss of its functional activities, such as the inhibition of TNF-α-induced immunoreactive MCP-1 from ASM cells and eosinophil chemotaxis. A similar loss of RANTES immunoreactivity and chemotactic function was observed with tryptase as a result of its proteolytic activity. However, tryptase had no proteolytic effect on other chemokines and cytokines that are known to be produced by human
ASM cells and to play a role in orchestrating airway inflammation, including MCP-1 (7), IL-8 (8), GM-CSF (11), RANTES (10), VEGF (34), and TGF-β1 (12). Tryptase also had no effect on eotaxin-2, which is not expressed in human ASM cells (35). It is interesting to note that another major mast cell protease chymase also selectively cleaved eotaxin, but had no effect on other chemokines screened in this study and did not alter the effect of tryptase on eotaxin and RANTES. The selective cleavage of eotaxin and RANTES is the first report that mast cell tryptase can regulate chemokines and their chemotactic activities through its proteolytic activity and adds to the complexity of the interactions between ASM cells and mast cells.

We also demonstrated that the cleavage of eotaxin by purified tryptase was due to the proteolytic activity of tryptase, rather than any other contaminating proteases or heparin by studies showing that 1) eotaxin cleavage was achieved with very low concentrations of tryptase (as low as 0.007 mU/ml); 2) rh-tryptase also cleaved eotaxin in the presence of heparin, whereas heparin alone had no effect; and 3) the cleavage of eotaxin by both tryptase and rh-tryptase was markedly reversed by the protease inhibitor leupeptin (inhibits tryptase), but not SBTI (inhibits proteases other than tryptase).

There seems to be a dissociation between the strong eotaxin immunoreactivity (20) and the eosinophil paucity in asthmatic ASM (1). However, the eotaxin immunoreactivity detected by immunohistochemistry (20) probably represents only functionally inactive eotaxin inside ASM cells, because eotaxin secreted outside ASM cells can be cleaved quickly by tryptase released from microlocalized mast cells and subsequently loses its immunoreactivity as well as its eosinophil chemotactic activity. The same applies...
to RANTES. Therefore, even if there is strong eotaxin and RANTES expression in asthmatic ASM, eosinophil paucity can still be observed in asthmatic ASM bundles because it mainly consists of the chemokines inside the cells. However, in addition to eotaxin and RANTES, there are other chemokines that may contribute to eosinophil migration into asthmatic ASM bundles and it is difficult to mimic the microenvironment of asthmatic ASM cells in in vitro experiments. Additional studies with asthmatic ASM are needed to verify the role of tryptase in eosinophil paucity in asthmatic ASM bundles.

Eotaxin is a selective ligand for the chemokine receptor CCR3, whereas RANTES binds to CCR3 as well as CCR1, CCR5, and CCR9. CCR3 is expressed in eosinophils (36), basophils (37), Th2 cells (38), and MCfts (39). Both eotaxin and RANTES exert chemotactic activities on mast cells through CCR3 (39). Tryptase cleavage of these chemokines may therefore provide a feedback regulation to prevent the excessive mast cell infiltration into human ASM bundle. However, there may be other, more important ASM cell-derived chemokines than eotaxin and RANTES that contribute to mast cell migration into ASM. Indeed, a recent study has demonstrated that tryptase-stimulated human ASM cells attract mast cells through the release of TGF-β1 in addition to SCF, and that both factors are localized to ASM in asthmatic airways (11). Another recent study has shown that the chemokine CXCL10 (IFN-γ-inducible protein 10) is expressed preferentially by asthmatic ASM cells, and its receptor CXCR3 is most abundantly expressed on mast cells in asthmatic ASM, suggesting that interactions between ASM-derived CXCL10 and mast cell expressed CXCR3 may play a key role in mast cell migration into the ASM bundles in asthma (40). Tryptase cleavage of eotaxin and RANTES may therefore not have an impact on mast cell microlocalization in asthmatic ASM.

Eosinophils have long been regarded as the fundamental effector cells in the pathogenesis of asthma. However, treatment with anti-IL-5 mAbs has no clinical benefit in asthma despite the profound reduction in circulating and airway lumen eosinophils (41, 42). The lack of efficacy of anti-IL-5 therapy and the lack of eosinophil reduction in circulating and airway lumen eosinophils (41, 42). IL-5 mAbs has no clinical benefit in asthma despite the profound reduction in circulating and airway lumen eosinophils (41, 42). Tryptase cleavage of eotaxin and RANTES may therefore not have an impact on mast cell microlocalization in asthmatic ASM.

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