Degradation of Monocyte Chemoattractant Protein-1 by Trypsin Co-Released in Immunoglobulin E-Dependent Activation of Primary Human Cultured Mast Cells

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Keywords

Human mast cells · Immunoglobulin E · Mast cell tryptase · Monocyte chemoattractant protein-1 · Mediator release

Abstract

Background: Mast cells are key immune effector cells which release chemokines, proteases, and other inflammatory mediators upon activation by immunological stimuli. The aim of this study was to investigate the effects of co-releasing proteases on the kinetics of release of the chemokine monocyte chemoattractant protein-1 (MCP-1) in immunoglobulin E (IgE)-mediated activation of human mast cells. **Methods:** Homogenous populations of mature and functional primary human mast cells were generated from CD34⁺ progenitors originated from buffy coats of healthy adult donors. The releases of MCP-1 from human mast cells in basal conditions

and in response to FcεRI cross-linking were assessed at different time points. The effects of different types of protease inhibitors on MCP-1 release from these mast cells under stimulated or unstimulated conditions were also investigated. **Results:** Cultured human mast cells released MCP-1 in basal conditions and its levels increased in a time-dependent manner. When stimulated by FcεRI cross-linking, the levels of MCP-1 detected in the medium gradually decreased over time after the initial peak induction. Such a decline in MCP-1 levels after IgE-dependent activation was completely prevented by pretreatment with a cocktail of protease inhibitors or the specific tryptase inhibitor APC366. **Conclusions:** Direct regulation of MCP-1 expression by co-release of tryptase in cultured human mast cells upon IgE-dependent activation demonstrates a role of the serglycin:serine protease axis in modulation of inflammatory reactions through proteolytic degradation of mediators such as chemokines.

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Introduction

Mast cells are key immune effector cells that mediate immunoglobulin E (IgE)-dependent immediate hypersensitivity reactions. Cross-linking of high-affinity IgE receptors (FcεRI) expressed on mast cells by allergen-specific IgE and allergens leads to degranulation with the immediate release of preformed mediators such as histamine, which is followed by *de novo* synthesis and release of lipid mediators and cytokines [1, 2]. Proteases, like histamine, are preformed and stored in secretory granules of mast cells until they are released through degranulation induced by IgE-dependent or IgE-independent mast cell activation. Through proteolytic modification of immunoreactive cytokines/chemokines in the immediate microenvironment in which mast cell degranulation occurs, the released proteases have been suggested to contribute significantly to the pathophysiological functions of mast cells in inflammation, wound healing, and innate immunity. Several recent studies using different mouse models have provided further evidence that serglycin proteoglycan and its associated proteases present in the granules of mast cells are involved in regulation of many inflammatory responses through either proteolytic activation or proteolytic degradation of cytokines that are present in the extracellular space [3–5].

We recently reported a novel protocol that can generate high yields of mature human connective tissue type (MC_{TC}) mast cells after only 6 weeks of culture [6]. In our studies on the release of cytokines and chemokines from human mast cells generated by our protocol, we observed that these cells spontaneously released the chemokine monocyte chemoattractant protein (MCP)-1 in basal conditions and that its release increased in a time-dependent manner. Interestingly, upon FcεRI-dependent activation, the levels of MCP-1 released from these human mast cells were found to gradually decrease over time after the initial peak induction. It has previously been shown in studies with purified human skin-derived mast cells that granular proteases, such as chymase and cathepsin G, released upon FcεRI cross-linking can degrade cytokines that are co-released from these activated mast cells [7]. Since MCP-1 is an important chemokine that regulates the migration and infiltration of leucocytes from the circulation to sites of inflammation [8], we were interested to investigate whether the reduction in MCP-1 levels observed after human mast cell activation was due to the proteolytic action of the proteases released by degranulation, which thereby limits the leucocyte-attracting property of tissue mast cells. In this study, we report

that the decline in MCP-1 levels detected in the medium after IgE-dependent cell activation was completely prevented by pretreatment with protease inhibitor cocktail or specific tryptase inhibitor APC366, suggesting the direct regulation of MCP-1 protein levels by tryptase co-released by primary human cultured mast cells (HCMC) upon IgE-dependent activation.

Materials and Methods

Materials

Iscove's modified Dulbecco's medium (IMDM), insulin-transferrin-selenium, 2-mercaptoethanol, penicillin-streptomycin, fetal bovine serum, stem cell factor (SCF), IL-3, and IL-6 were purchased from Invitrogen. IL-4 and IL-9 were purchased from PeproTech. Human myeloma immunoglobulin (IgE) was purchased from Calbiochem. Bestatin, chymostatin, substance P, and anti-human IgE (AE) were purchased from Sigma-Aldrich, and 1× protease inhibitor cocktail tablets were purchased from Roche. APC366 was purchased from Tocris Bioscience.

Generation of HCMC

Primary human mast cells with characteristics of the connective tissue type (MC_{TC}) phenotype were generated using a protocol previously described by our group [6]. Briefly, CD34⁺ progenitors isolated from fresh adult peripheral blood buffy coat using the MACS system (Miltenyi Biotec) were cultured in complete IMDM (with 1× insulin-transferrin-selenium, 1× penicillin-streptomycin, 50 μM 2-mercaptoethanol, and 0.1% BSA) with 200 ng/mL SCF, 100 ng/mL IL-6, and 1 ng/mL IL-3 for 1 week. Subsequently, the spent medium was replaced by complete IMDM with 200 ng/mL SCF, 100 ng/mL IL-6, and 15 ng/mL IL-9 for another week. For these initial 2 weeks of culturing, cells were incubated in hypoxic conditions (5% CO₂, 5% O₂, and 37°C). After the first 2 weeks, the spent medium was replaced by complete IMDM with 200 ng/mL SCF and 100 ng/mL IL-6 weekly for another 2 weeks. At week 5, the spent medium was replaced by complete IMDM with 200 ng/mL SCF, 100 ng/mL IL-6, and 10 ng/mL IL-4. From week 6 and thereafter, cells were cultured in complete IMDM with 100 ng/mL SCF and 50 ng/mL IL-6. From week 3 onwards, these cultures were incubated in normoxic conditions (5% CO₂, 21% O₂, and 37°C). After week 6, fully mature and homogenous populations of HCMC were generated and ready for functional studies.

Measurement of Histamine Release

The release of histamine from mature cultured human mast cells (cultured for ≥6 weeks) was measured as previously described [6]. Briefly, the cells were harvested, counted, seeded, and sensitized with 0.5 μg/mL human myeloma IgE overnight in complete IMDM with 100 ng/mL SCF and 50 ng/mL IL-6. The cells were then washed once in PBS and resuspended in full HEPES buffer (137 mM NaCl, 5.56 mM glucose, 12 mM HEPES, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, and 1 mM CaCl₂ at pH 7.4) with 0.03% HSA and then challenged by anti-IgE or substance P for 30 min at 37°C. The reaction was stopped by the addition of ice-cold buffer and chilling on ice. Samples of cell-free supernatant and corresponding cell pellets were then collected by cen-

trifugation at 4 °C. Cells incubated in buffer alone served as the controls for spontaneous histamine release. The histamine contents in mast cell pellets and supernatants were measured spectrofluorometrically using the Bran+Luebbe AutoAnalyzer 3. Histamine release was expressed as a percentage of the total cellular contents of histamine (the sum of the supernatant content and the cell pellet content) that were released into the supernatant (histamine release, %).

Measurements of Releases of MCP-1 and IL-8

IgE-sensitized or naive mature cultured human mast cells were washed and resuspended in complete IMDM containing 100 ng/mL SCF, 50 ng/mL IL-6, and 10% fetal bovine serum. The cells were stimulated with 0.25 µg/mL anti-IgE at 37 °C overnight and the culture medium was then harvested with cells removed by centrifugation at 4 °C. For time course experiments, media were harvested at selected time points after activation with anti-IgE and frozen at -80 °C. The amounts of MCP-1 and IL-8 released into the culture medium were measured using ELISA (BD) and presented as ng/10⁶ cells.

Protease Inhibitor Studies

For studies employing protease inhibitors, IgE-sensitized cultured human mast cells were stimulated with 0.25 µg/mL anti-IgE at 37 °C for 6 h. The culture media were then harvested and treated with different protease inhibitors or water under the following experimental conditions:

1. Frozen 6-h reaction: samples were harvested at the 6th hour and immediately frozen at -80 °C.
2. Water control: samples were harvested at the 6th hour and treated with water and kept at 37 °C for another 16 h.
3. Protease inhibitor cocktail: samples were harvested at the 6th hour and treated with 1× Roche protease inhibitor cocktail at 37 °C for another 16 h.
4. APC366 (specific inhibitor of mast cell tryptase): samples were harvested at the 6th hour and treated with 500 µg/mL APC366 at 37 °C for another 16 h.
5. Bestatin (specific inhibitor of metalloprotease): samples were harvested at the 6th hour and treated with 500 µM bestatin at 37 °C for another 16 h.
6. Chymostatin (specific inhibitor of chymotrypsin): samples were harvested at the 6th hour and treated with 100 µg/mL chymostatin at 37 °C for another 16 h.
7. Frozen 16-h reaction: samples were harvested at the 16th hour and immediately frozen at -80 °C.

Quantitative PCR

The primer sequences were as follows:

MCP-1: forward 5'-CGCTCAGCCAGATGCAATCAA-3'; reverse 5'-GACACTTGCTGCTGGTGATTC-3'.

GAPDH: forward 5'-AGGTGAAGGTCGGAGTC-3'; reverse 5'-GGAAGATGGTGATGGGATT3'.

Real-time qPCR was performed using SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara) with the 7900HT Fast Real-Time PCR System (ABI). Cycle thresholds (Ct) for MCP-1 and GAPDH transcripts were determined. Relative fold changes between the expression of the genes of interest in treated and untreated samples were determined via the 2^{-(ddCt)} method. Each treatment sample was assessed using 3 different batches of primary mast cell cultures. All of the samples were repeated in triplicate.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (version 5.02). The levels of release of MCP-1 and IL-8 determined by ELISA are presented as means ± SEM (in ng/10⁶ cells). Statistical significance was determined using a paired *t* test, an unpaired *t* test or a one-way ANOVA where appropriate.

Results

Spontaneous Release of MCP-1 from HCMC and Its Reduction by Anti-IgE Stimulation

Anti-IgE stimulation induced degranulation in our HCMC, resulting in the immediate release of histamine (Fig. 1a; unstimulated spontaneous release: 4.0 ± 1.1%; anti-IgE stimulated release: 39.0 ± 2.0%). Release of MCP-1 from IgE-sensitized HCMC was also readily detected 16 h after anti-IgE stimulation (Fig. 1b; 8.6 ± 1.2 ng/10⁶ cells). Interestingly, significantly higher levels of MCP-1 were spontaneously released from these IgE-sensitized HCMC that had not been stimulated by anti-IgE (Fig. 1b; 58.0 ± 3.3 ng/10⁶ cells). Indeed, similar levels of MCP-1 were released by naive non-IgE sensitized HCMC in the absence or presence of anti-IgE (Fig. 1b; 57.9 ± 1.9 and 53.4 ± 3.4 ng/10⁶ cells, respectively). These data suggest that MCP-1 was spontaneously released from HCMC into the medium, while FcεRI activation induced a significant decrease in the levels of MCP-1 detected in the medium. On the other hand, IL-8 release from HCMC was only detected after FcεRI cross-linking (Fig. 1b; unstimulated: 0.2 ± 0.1 ng/10⁶ cells; anti-IgE stimulation: 57.9 ± 11.5 ng/10⁶ cells), and the released levels were similar to those observed in our previous study [6], thus implying that these HCMC responded to IgE-dependent cell activation in the typical expected manner.

Nonimmunological activation by substance P resulted in degranulation which was represented by a significant histamine release (Fig. 1c; spontaneous release: 3.4 ± 0.3%, 1 µM substance P: 71.5 ± 5.3%, 10 µM substance P: 71.5 ± 2.5%). Similar to anti-IgE induced activation, the levels of spontaneously released MCP-1 were significantly reduced following activation induced by substance P (Fig. 1d; unstimulated: 70.6 ± 3.1 ng/10⁶ cells; 1 µM substance P: 12.9 ± 1.7 ng/10⁶ cells; 10 µM substance P: 7.2 ± 0.8 ng/10⁶ cells; *n* = 3).

Time Course of Release of MCP-1 from HCMC under Basal Conditions and in Response to Anti-IgE Stimulation

To elucidate the mechanisms underlying the decrease in MCP-1 release induced by anti-IgE stimulation ob-

Fig. 1. Release of histamine and cytokines from human cultured mast cells in basal condition and in response to activation by anti-IgE or substance P. **a** Release of histamine in basal conditions and in response to 0.25 $\mu\text{g/mL}$ anti-IgE ($n = 5$). **b** Release of MCP-1 and IL-8 in basal conditions and in response to 0.25 $\mu\text{g/mL}$ anti-IgE stimulation ($n = 4$). **c** Release of histamine in basal conditions and in response to substance P (SP) stimulation ($n = 3$). **d** Release of MCP-1 in response to stimulation by substance P ($n = 3$). All values are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by paired t test when compared with corresponding basal values obtained with unstimulated cells.

served with HCMC, MCP-1 release was assessed 1, 2, 3, 6, 9, 16, and 24 h after activation. As shown in Figure 2a, MCP-1 protein was spontaneously released from HCMC and steadily accumulated in the culture medium in a time-dependent manner from the 1st to the 24th hour. In contrast, as illustrated in Figure 2b, anti-IgE activated HCMC released higher levels of MCP-1 compared to those in the basal state at the 1st hour (5.8 ± 1.5 vs. 2.3 ± 0.6 ng/ 10^6 cells), and the induced levels of MCP-1 became increasingly higher at the 2nd hour (16.3 ± 5.0 ng/ 10^6 cells) and 3rd hour (27.3 ± 7.3 ng/ 10^6 cells) and peaked at the 6th hour (29.8 ± 10.9 ng/ 10^6 cells) (Fig. 2a). However, the levels of MCP-1 started to decrease from the 9th hour (24.8 ± 7.7 ng/ 10^6 cells) and were lower than those of the corresponding spontaneous release levels (Fig. 2a) at the 16th hour (9.1 ± 5.8 vs. 23.3 ± 5.4 ng/ 10^6 cells) and the 24th hour (8.8 ± 4.1 vs. 32.0 ± 6.2 ng/ 10^6 cells). These data suggested that anti-IgE stimulation in-

duced a rapid increase in the release of MCP-1 into the medium.

On the other hand, the gradual decline in MCP-1 levels starting at the 6th hour after anti-IgE stimulation raised the possibility that MCP-1 expression might be down-regulated in the later time period of anti-IgE stimulation. To assess such a possibility, we performed quantitative PCR to measure mRNA expression of MCP-1 in control and anti-IgE stimulated HCMC (Fig. 2c). MCP-1 mRNA levels were 1.8-fold higher in anti-IgE stimulated cells than in control cells after 30 min ($p < 0.0001$), suggesting that MCP-1 gene transcription increased significantly in response to anti-IgE stimulation as early as 30 min. However, after 24 h, there was no significant difference in mRNA levels of MCP-1 between stimulated and control cells ($p = 0.5181$), suggesting that the gradual decline in MCP-1 release after a prolonged period could not be attributed to a decrease in de novo MCP-1 synthesis.

Fig. 2. Time course of MCP-1 release from human cultured mast cells. **a** Under basal conditions. **b** Following activation by 0.25 $\mu\text{g/mL}$ anti-IgE. All values are means $\pm$ SEM ($n = 3$). ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA with Dunn's multiple comparisons test when compared with the value at 1 h. **c** Expression of MCP-1 mRNA in human cultured mast cells in basal conditions and after 30 min and 24 h of stimulation with 0.25 $\mu\text{g/mL}$ anti-IgE. All values are means $\pm$ SEM ($n = 3$). **** $p < 0.0001$ by unpaired t test when compared with the corresponding basal level.

Fig. 3. Effects of different protease inhibitors on levels of MCP-1 release from human cultured mast cells. **a** Under basal conditions. **b** In response to 0.25 $\mu\text{g/mL}$ anti-IgE stimulation. All values are means $\pm$ SEM ($n = 5-9$). * $p < 0.05$ and ** $p < 0.01$ by unpaired t test when compared with the control frozen 6-h sample.

Degradation of MCP-1 by Trypsin Co-Released from Anti-IgE-Activated HMC

To assess whether MCP-1 released from HMC was degraded by preformed granular proteases co-released after activation of the high-affinity IgE receptors, differ-

ent protease inhibitors were tested for their abilities to inhibit the decrease in MCP-1 levels. IgE-sensitized HMC with or without anti-IgE activation were incubated at 37°C for 6 h. Cell-free supernatants were then collected and subsequently treated with water (vehicle)

or different protease inhibitors for an additional 16 h at 37 °C. MCP-1 levels in the treated supernatants were then determined. As a control, MCP-1 protein released by unstimulated cells into the medium was harvested at the 6th hour and frozen immediately for subsequent analysis. As shown in Figure 3a, for all MCP-1 samples derived from nonactivated HCMC, no MCP-1 degradation was observed among all of the 6th hour samples, regardless of their subsequent treatments (range: 10.5 ± 4.8 to 14.8 ± 3.3 ng/ 10^6 cells). In comparison, samples harvested from unstimulated cells at the 16th hour showed significantly higher MCP-1 levels than samples at the 6th hour (55.8 ± 13.4 vs. 14.7 ± 3.1 ng/ 10^6 cells, respectively). On the other hand, in the absence of any protease inhibitor treatment (i.e., water vehicle control), the MCP-1 levels in samples collected from anti-IgE-activated HCMC decreased significantly after the 16th hour with incubation at 37 °C when compared with the 6th-hour sample (10.1 ± 2.3 vs. 28.9 ± 5.1 ng/ 10^6 cells, respectively; Fig 3b). However, MCP-1 levels were not affected in those anti-IgE-activated HCMC samples that were treated with either the protease inhibitor cocktail (27.2 ± 4.9 ng/ 10^6 cells) or 500 µg/mL APC366, a trypsin-specific inhibitor (26.5 ± 5.1 ng/ 10^6 cells). In contrast, MCP-1 degradation was not inhibited by 500 µM bestatin, a metalloprotease inhibitor (6.2 ± 1.9 ng/ 10^6 cells), or 100 µg/mL chymostatin, a chymase inhibitor (9.7 ± 2.5 ng/ 10^6 cells).

Discussion

We recently developed a novel protocol for generating large homogenous populations of mature and functional HCMC from buffy coats that can respond to different mast cell stimuli, including substance P, by releasing different preformed and newly synthesized mediators and cytokines that are characteristic of connective tissue-type mast cells (MC_{TC}) [6]. In this study, we showed that nonactivated HCMC spontaneously released the chemokine MCP-1 in basal conditions and that the released MCP-1 levels increased in a time-dependent manner. On the other hand, an enhanced release of MCP-1 from HCMC was induced by FcεRI cross-linking but the elevated MCP-1 levels gradually decreased over time to levels that were lower than those observed with basal spontaneous release (Fig. 1, 2). The reduction of MCP-1 levels was also observed when these mast cells were activated with substance P, which bypasses FcεRI to induce degranulation, thus suggesting

that the reduced MCP-1 levels are linked to the granular contents co-released at the time of mast cell activation. Similar findings on spontaneous and activated release of MCP-1 from human mast cells were reported in a recent study in which HCMC with the MC_{TC} phenotype were generated using a culturing protocol that is entirely different from ours [9], suggesting that our observations are most unlikely an experimental artifact induced by specific culturing conditions employed in our culturing protocol. Moreover, gene expression studies (Fig. 2b) showed that *MCP-1* mRNA levels were elevated shortly after FcεRI cross-linking (30 min), while they returned to normal levels after a prolonged period of time (24 h).

MCP-1 is a potent monocyte attractant and has been reported to be involved in various diseases. Migration of monocytes from the blood stream across the vascular endothelium is required for routine immunological surveillance of tissues and in response to inflammation [10]. Mast cells are known to secrete the MCP-1 protein, but they are also recruited to the inflammatory sites by this chemokine. Thus, an investigation on the feedback mechanism for the regulation of MCP-1 levels may help us to gain some insights into the leucocyte-attracting property of tissue mast cells.

Using a panel of inhibitors of trypsin and chymase, we showed that the gradual decrease in MCP-1 levels was mediated by proteolytic degradation of the MCP-1 protein by trypsin that was co-released from the activated human mast cells. The decline in MCP-1 levels detected in the medium after IgE-dependent cell activation was completely prevented by pretreatment with protease inhibitor cocktail or the specific trypsin inhibitor APC366, but not by inhibitors of chymase and metalloprotease, suggesting the direct regulation of MCP-1 protein levels by trypsin co-released by HCMC upon IgE-dependent activation (Fig. 3). Therefore, the following scenario that describes the mechanisms of such actions can be proposed: HCMC slowly but spontaneously release MCP-1 protein which accumulates stably in the medium in a time-dependent manner. FcεRI cross-linking of these cells with anti-IgE induces an additional increase in MCP-1 release. However, the time-dependent proteolytic activity of trypsin co-released during degranulation can gradually degrade MCP-1 protein that has accumulated in the medium, thus causing the MCP-1 levels to drop 6 h after degranulation.

Several recent studies using different mouse models have suggested that serglycin proteoglycan and its associated proteases present in the granules of mast cells are

involved in regulation of many inflammatory responses [3–5]. Depending on the experimental settings, the release of these proteases from mast cells upon degranulation can have either beneficial or detrimental effects on cell surroundings [11–19]. The mechanisms by which such proteases released by mast cells regulate inflammation involve either proteolytic activation or proteolytic degradation of cytokines in the extracellular environment. Several reports have shown that mast cell serine proteases can proteolytically activate cytokines and chemokines [20–24]. On the other hand, a recent report demonstrated that activated cultured mouse peritoneal mast cells can regulate levels of IL-3, IL-6, IL-13, IL-17A, and eotaxin by releasing serglycin-dependent serine proteases, which mediate the proteolytic degradation of these cytokines and chemokines [25]. Previous in vitro studies with purified human skin-derived mast cells have shown that chymase and cathepsin G released upon FcεRI cross-linking degrade cytokines, such as IL-6 and IL-13, that are co-released from these activated mast cells [7]. In this study, we have further demonstrated that tryptase released from HCMC following FcεRI cross-linking can degrade the MCP-1 co-released from the stimulated mast cells. Thus, our data are consistent with the view that human mast cells release proteases that can degrade cytokines that are co-released during

cell activation. Taken together, the data obtained from studies of both mouse and human mast cells are consistent with a role of the serglycin:serine protease axis in the downregulation of levels of inflammatory cytokines [3–5].

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Statement of Ethics

The use of human buffy coat preparations for the culturing of human mast cells was approved by The Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee.

Disclosure Statement

Some of the data described in this report were included in a patent application filed with the US Patent Office.

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