Cysteinyl Leukotrienes Enhance the Degranulation of Bone Marrow-Derived Mast Cells through the Autocrine Mechanism

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The cysteinyl leukotrienes (LTs), LTC₄, LTD₄, and LTE₄, are potent inflammatory mediators and are involved in allergic reactions, such as bronchoconstriction, eosinophilic inflammation, and allergic cell proliferation. The present study aimed to elucidate the role of constitutively produced cysteinyl LTs in mast cell activation. We used a newly developed quantification method based on mass spectrometry to detect cysteinyl LTs in the cultured medium of mouse bone marrow-derived mast cells (BMMCs), which were obtained by interleukin (IL)-3-conditioned culture of mouse bone marrow. BMMCs were stimulated with immunoglobulin (Ig) E and antigen (IgE/Age) or lipopolysaccharide for 1 or 24 h. This new quantification method revealed that unstimulated BMMCs produced and secreted LTB₄ and LTE₄ after 24 h of incubation. The treatment of unstimulated BMMCs for 2 h with montelukast, an antagonist of a cysteinyl LT receptor, CysLT₁, resulted in the suppression of a downstream signaling event of this receptor, i.e., the decrease in phosphorylation of extracellular responsive kinases. Thus, cysteinyl LTs constitutively simulate BMMCs through the CysLT₁ receptor in an autocrine manner. Treatment of BMMCs for 3 weeks with montelukast, which caused long-term inhibition of the autocrine cysteinyl LT-derived signal, significantly attenuated the IgE/Age-dependent degranulation, as judged by the decrease in the release of β-hexosaminidase, an enzyme contained in the granules, whereas the production of cytokines, such as IL-6 and tumor necrosis factor-α, were largely unaffected. In conclusion, an autocrine signal derived from constitutively produced cysteinyl LTs predisposes mast cells to the degranulation upon allergic stimulation. ———— autocrine; CysLT₁; cysteinyl leukotriene; mast cell; montelukast.


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CysLT2 (Mellor et al. 2001; Bautz et al. 2001; Figueroa et al. 2003; Mellor et al. 2003). Mice lacking the LTC₄ synthetase, an enzyme that is required for the synthesis of all cysteinyll LTs, show markedly attenuated mast cell hyperplasia in response to an allergenic challenge (Kim et al. 2006); this suggests that CysLT1 and/or CysLT2 play a critical role in the development or migration of mast cells in vivo.

Mast cells are critical for the initiation of allergic diseases: they detect allergens, release early inflammatory mediators such as histamine, and mediate the type 2 helper T cell (Th2) response by releasing cytokines. In light of the importance of lipid mediators in the onset of allergic reactions, we have investigated their roles in mast cell activation by using bone marrow-derived, interleukin (IL)-3-dependent mast cells (BMMCs) (Kaneko et al. 2008). In the initial phase of the present study, we detected cysteinyll LTs in the cultured medium of unstimulated BMMCs by using a new method based on mass spectrometry. This finding, in turn, raised a new question about the biological significance of the constitutive production of cysteinyll LTs in the function of BMMCs. The present findings provide a new insight into the important role of CysLT1-mediated signals in mast cell exocytosis.

MATERIALS AND METHODS

Mast cell culture and activation

Bone marrow cells were prepared from 2 to 3 month-old C57BL/6 and MRL/Mp mice (Charles River Breeding Laboratories, Yokohama, Japan). BMMCs were obtained by a long-term culture (>3 weeks) of mouse bone marrow cells in the RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, non-essential amino acids, 50 μM 2-mercaptoethanol (SIGMA, St. Louis, MO), and 5 ng/ml murine IL-3 (R&D Systems, Minneapolis, MN). BMMCs (10⁵/200 μl) were activated with 1 mg/ml trinitrophenyl (TNP)-specific immunoglobulin (Ig) E (TNP-IgE) and 0.1-30 ng/ml of TNP-conjugated ovalbumin (TNP-OVA) (fraction VII, SIGMA). Lipopolysaccharide (LPS; Escherichia coli 055:B5; SIGMA) was used for stimulation at a concentration of 0.1 or 1 μg/ml. An antagonist of CysLT1, montelukast, which was kindly donated by Merck Co. Ltd., was supplemented into the culture at 1 or 10 μM for 2 h or 3 weeks before activation. During the long-term culture of BMMCs with montelukast, the medium was replaced twice a week with fresh medium supplemented with montelukast.

Liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis

The simultaneous quantification of LTs was performed using the cultured medium collected at 1 h or 24 h after the stimulation. LTB₄-d₅, LTD₄-d₅, LTE₄-d₅, and LTE₄-d₅ (BIOMOL, Plymouth Meeting, PA) (each 2 ng) were added into the cultured medium (0.1 ml) as internal standards. The sample was acidified and passed through an Empore C18 HD disk cartridge (3 M Industry, St. Paul, MN). The bound fraction was collected in acetonitrile-water (3:7, v/v; 1 ml) as the LT-enzriched fraction. After evaporating the solvent, the residue was reconstituted in the mobile phase (40 μl), sonicated for 30 s, and filtered. The reconstituted sample was transferred to an autosampler vial; 10 μl of the sample was subjected to the LC/MS-MS analysis. The high-performance liquid chromatography (HPLC) was performed in a Capcell Pak C18 MGII column (1.5 x 150 mm; 5 μm) (Shiseido, Tokyo, Japan) using gradient elution with acetonitrile (solvent A) and 5 mM ammonium acetate (solvent B) at a flow rate of 200 μl/min at 40°C. The gradient was set up as follows: the fraction of solvent A was increased from 23% to 53% in 3 min and then maintained at 53% for 6 min. The LC/MS/MS analysis was performed using a TSQ Quantum Ultra Triple quadrupole mass spectrometer (Thermo Fisher, Waltham, MA) equipped with an electrospray ion source and operated in the negative-ion mode. Using selected reaction monitoring (SRM), transitions of m/z 335 to 195, m/z 624 to 272, m/z 495 to 177, and m/z 438 to 333 were used for the measurement of LTB₄-d₅, LTD₄-d₅, LTE₄-d₅, and LTE₄-d₅, respectively.

Activation of extracellular signal-regulated kinases (ERKs)

The activities of ERKs 1/2, which are known to be phosphorylated on receiving signals via CysLT1, were monitored by the detection of their phosphorylated form with western blot analysis and flow cytometry. For the western blot analysis, BMMCs (5 x 10⁵) were suspended on ice in 100 μl radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1 mM phosphatase inhibitor cocktail (Sigma). The postnuclear fraction was mixed with sodium dodecyl sulphate (SDS) sample buffer and denatured in boiled water for 5 min. A sample volume containing 5 x 10⁵ cells was fractionated on a polyacrylamide gel (12.5%) containing SDS and transferred to a nitrocellulose membrane. Phosphorylation of ERK1/2 was monitored by the detection of the corresponding bands using antibodies against threonine 202 and tyrosine 204 (Cell Signaling, Danvers, MA) which were used as the primary antibodies. In the flow cytometry, BMMCs (10⁵) were treated with montelukast or the mitogen-activated protein kinase kinase-1 inhibitor PD98059 for 2 h and fixed in phosphate-buffered 2% paraformaldehyde for 30 min at room temperature; this was followed by further incubation in 90% ice-cold ethanol for 15 min to allow the antibodies to permeate the cells. The primary antibody anti-phospho-ERK1/2 antibody (T202/Y204) (Cell Signaling Tech, Danvers, MA) and the secondary antibody Alexa 594-conjugated anti-rabbit antibody were serially used for immunostaining.

Cytokine release and degranulation of BMMCs

The concentrations of IL-6 and tumor necrosis factor (TNF)-α in the cultured medium collected at 6 h after the stimulation were determined by enzyme-linked immunosorbent assay (ELISA) kits (BD PharMingen, San Diego, CA). The degranulation response of BMMCs was quantified by determining the fraction of released β-hexosaminidase or histamine in the cultured medium as previously described (Ono et al. 1999). Histamine concentration was measured using the histamine ELISA kit (Neogen, Lexington, KY). The percent degranulation of BMMC was estimated by the following formula: 100 x (β-hexosaminidase activity or histamine in the supernatant fraction)/ (total β-hexosaminidase activity or histamine in the cellular and supernatant fractions).

Statistical analysis

The difference between 2 mean values was evaluated by the
RESULTS AND DISCUSSION

Constitutive production of cysteinyl LTs and LTB₄ in the BMMCs

We have previously shown that the autocrine release of constitutively produced prostanooid species significantly contributes to mast cell activation (Kaneko et al. 2008). In this previous study, we also demonstrated the suppressive effect of a 5-lipoxygenase (5-LO) inhibitor, nordihydroguaiaretic acid, on the cytokine responses mediated by BMMCs. These findings have provided a better understanding of the role of 5-LO metabolites and prostanoids in BMMC activation. To identify the individual LTs liberated from the BMMCs and to quantify each LT, we developed a new highly sensitive and specific method for the simultaneous determination of the amounts of LTB₄, LTC₄, LTD₄, and LTE₄ in the cultured medium. We used 3 culture conditions: no treatment, treatment with LPS (100 ng/ml), and treatment with IgE plus antigen (IgE/Ag) (1 ng/ml TNP-OVA). All the LTs were detected in the culture medium 1 h after the treatment with IgE/Ag, but LTs were not detected after the treatment with LPS (Table 1), despite that LPS increased cytokine production in BMMCs under certain conditions (Kaneko et al. 2008). Notably, 24 h after the treatment, LTB₄ and LTE₄ were detected in the culture media of untreated BMMCs and IgE/Ag-stimulated BMMCs. LTC₄ and LTD₄ were not detected in the cultured medium at 24 h probably because they were metabolically converted to LTE₄. These findings indicate that BMMCs synthesize LTB₄ and other cysteinyl LTs independent of stimulation.

At a concentration of 10 nM, cysteinyl LTs are known to influence the signaling events in mast cells: calcium influx, the activation of the mitogen-activated protein kinase pathway, production of cytokines and chemokines, and internalization of the c-KIT receptor (Jiang et al. 2007). In the present study, the LTE₄ concentration in the cultured media after 24 h was as high as 170 nM, which is comparable to the LTE₄ concentrations that generally initiate signaling events. These findings suggest an interesting possibility that the autocrine secretion of constitutively expressed cysteinyl LTs influences BMMC functions.

Constitutive cysteinyl LTs in BMMC culture activate ERKs via CysLT1

We examined whether cysteinyl LTs affected the signal transduction in BMMCs under the untreated condition. CysLT1-mediated signals were assessed by comparing the active state of ERK1/2, which is reflected by pERK1/2, in the presence or absence of the CysLT1 antagonist montelukast, which affects the signaling events downstream of CysLT1. pERK1/2 was detected using western blot analysis with an anti-ERK1/2 antibody (Fig. 1A) and flow cytometric analysis (Fig. 1B). We found that the amount of pERK1/2 after treatments with 1 or 10 μM montelukast was lower than the amount in untreated cells. BMMC proliferation was not affected by treatment with 1 μM montelukast, but it was marginally suppressed by treatment with 10 μM montelukast (data not shown). These findings suggest that the constitutively produced cysteinyl LTs present in the untreated condition participated in CysLT1-mediated signal transduction in BMMCs.

Constitutive CysLT1-mediated signals affect the degranulation of BMMCs

In order to test the hypothesis that the CysLT1-mediated signals influence BMMC functions in an autocrine manner, we quantified the degranulation (Fig. 2A-E) and cytokine production (Fig. 2F and G) under 3 conditions: no treatment, treatment with montelukast (1 or 10 μM) for 2 h, and treatment with montelukast for 3 weeks. To distinguish

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTB₄</th>
<th>LTC₄</th>
<th>LTD₄</th>
<th>LTE₄</th>
</tr>
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<tbody>
<tr>
<td>For 1 h</td>
<td></td>
<td></td>
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<tr>
<td>IgE/Ag</td>
<td>8.15 (7.67)</td>
<td>6.03 (8.56)</td>
<td>2.94 (2.08)</td>
<td>33.1 (28.2)</td>
</tr>
<tr>
<td>For 24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>untreated</td>
<td>2.21 (1.90)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>14.7 (6.95)</td>
</tr>
<tr>
<td>IgE/Ag</td>
<td>5.09 (2.87)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>34.2 (27.4)</td>
</tr>
</tbody>
</table>

Each value denotes a mean (standard deviation: S.D.) of the concentration of three samples in a single experiment, each of which was independently treated. BMMCs were stimulated without (untreated) or with 0.1 μg/ml LPS (LPS), or 1 ng/ml TNP-OVA following IgE sensitization (IgE/Ag). Culture supernatants were collected at 1 h (For 1 h) or 24 h (For 24 h) after stimulation.

LT, leukotriene; n.d., not detected.
Autocrine Leukotrienes Regulate Mast Cell Exocytosis

Mast cells are known to robustly produce cysteinyl LTs upon inflammatory stimulation and bear the cysteinyl LT receptors CysLT1 and CysLT2. The role of autocrine secretion of constitutively produced cysteinyl LTs in mast cell functions has been shown by using BMMCs grown in the presence of stem cell factor (SCF) and IL-4 (Jiang et al. 2006). This previous study has shown that the genetic depletion of CysLT1 in BMMCs or treatment of BMMCs with MK571, a CysLT1 antagonist, markedly attenuates their proliferation with reduced phosphorylation of ERK1/2. The present study revealed that CysLT1 antagonism has little effect on the proliferation of BMMCs. The difference between the present and previous results with respect to the effect of CysLT1-mediated signals on cell proliferation is probably attributable to the difference in the growth conditions. SCF and IL-4 were used in the previous study, while IL-3 was used in the present study. Nevertheless, cysteinyl LTs can be considered to be occasionally or constitutively present in the mast cell microenvironment independent of the growth condition.

It has been shown that transient signals via CysLT1 and CysLT2 lead to activation of mast cell that revealed by calcium influx, activation of the mitogen-activated protein kinase pathway, and production of cytokines and chemokines (Mellor et al. 2001; Lin and Boyce, 2005; Paruchuri et al. 2008). A previous report has revealed an effect of montelukast on mast cell function other than its primary effect, i.e., CysLT1 antagonism (Ramires et al. 2004). In this previous study, montelukast was shown to serve as an inhibitor of 5-LO, which is an enzyme essential for the in vivo synthesis of leukotriene species. In contrast to an increasing amount of information regarding the effects of the temporal activation of CysLT1 and/or CysLT2, little information is available regarding the chronic effects of CysLT1- and/or CysLT2-mediated signals. We showed for the first time the remarkable chronic effects of these signals on mast cell activation. These constitutive signals seem to be involved in the initiation of degranulation but not cytokine production in mast cells. This fact clearly depicts a distinct difference between the effects of temporal and chronic CysLT1-

Fig. 1. Effect of CysLT1 inhibition by montelukast on ERK1/2 phosphorylation in BMMCs. (A) pERK1/2 detected by western blot analysis with anti-pERK1/2 antibody. The level of pERK1/2 decreased after the incubation of BMMCs with 1 μM (lane 2) or 10 μM (lane 3) montelukast for 2 h as compared to the levels in untreated BMMCs (lane 1). The level of pERK1/2 induced by IgE/Ag stimulation is also shown (lane 4). Values at the bottom of the figure represent the fraction of the signal intensity of the treated samples compared to that of the untreated sample. (B) Flow cytometric detection of pERK1/2. Treatment with montelukast (Mont. 1 or 10 μM) or PD98059 (PD. 10 μM) reduced the level of pERK1/2. 1st Ab (―), omission of incubation with primary antibody; N.T., not treated. We confirmed these changes in the level of pERK1/2 by performing another experiment involving western blot and flow cytometric analysis.

the effects of the treatment on the early and late stages of BMMC development, the treatments were administered on a preparation of immature BMMCs (at the third week of culture) and a preparation of mature BMMCs (at the sixth week of culture). The degranulation induced on IgE/Ag stimulation was assessed by determining the fraction of released β-hexosaminidase, an enzyme present in granules (Fig. 2A and B), or histamine (Fig. 2C–E). The β-hexosaminidase assays revealed that the long-term treatment significantly suppressed the degranulation, whereas the short-term treatment did not suppress it. The histamine assays revealed the same tendency as observed in the β-hexosaminidase assays, although statistical significance was not evident probably owing to the small number of samples examined. Further, the results were similar for both mature and immature BMMCs. The total amounts of β-hexosaminidase (data not shown) and histamine (Fig. 2C–E) were not altered in either condition. These results indicate that the efficacy of CysLT1 receptor antagonism critically differs according to the period of the treatment. In contrast, the production of IL-6 (Fig. 2F) and TNF-α (Fig. 2G) after IgE/Ag stimulation was not influenced by CysLT1 antagonism. The expression of the c-KIT and Fc receptors for IgE (Fce RI) remained unaltered after the long-term treatment (data not shown). Collectively, these findings show that in BMMCs, constitutively produced cysteinyl LTs play a substantial role in the development of exocytosis, which is a fundamental function of mast cells.

Mast cells are known to robustly produce cysteinyl LTs upon inflammatory stimulation and bear the cysteinyl LT receptors CysLT1 and CysLT2. The role of autocrine secretion of constitutively produced cysteinyl LTs in mast cell functions has been shown by using BMMCs grown in the presence of stem cell factor (SCF) and IL-4 (Jiang et al. 2006). This previous study has shown that the genetic depletion of CysLT1 in BMMCs or treatment of BMMCs with MK571, a CysLT1 antagonist, markedly attenuates their proliferation with reduced phosphorylation of ERK1/2. The present study revealed that CysLT1 antagonism has little effect on the proliferation of BMMCs. The difference between the present and previous results with respect to the effect of CysLT1-mediated signals on cell proliferation is probably attributable to the difference in the growth conditions. SCF and IL-4 were used in the previous study, while IL-3 was used in the present study. Nevertheless, cysteinyl LTs can be considered to be occasionally or constitutively present in the mast cell microenvironment independent of the growth condition.

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Fig. 2. Blocking of the exocytotic response of BMMCs to crosslinking of FcεRI via CysLT1 inhibition. (A, B) Effect of treatment with 10 μM montelukast on β-hexosaminidase release from immature BMMCs (A) or mature BMMCs (B), which were stimulated at the end of the third or sixth week, respectively, of bone marrow culture. The average percent release of triplicate samples is shown. (–), no treatment (triangle); short, short-term (2 h) treatment (filled circle); long, long-term treatment (open circle). These results were reproduced in a second experiment performed using independently prepared samples. (C–E) Effect of treatment with 1 μM montelukast on total (cellular and released) histamine (C), released histamine (D), and percent fraction of the released histamine (E). The results obtained from 3 independent BMMC preparations are shown. B6-1, a B6 mouse (filled square); B6-2, another B6 mouse (filled triangle); MRL, an MRL mouse (open diamond). (F, G) Effects on the production of IL-6 (F) and TNF-α (G) in BMMCs. The same samples as in B were used for these measurements. Bars represent the standard deviation (S.D.) of triplicate samples. Asterisk(s) at each dose of antigen indicates (indicate) significant difference between (–) and long. *p < 0.05; **p < 0.01.
and/or CysLT2-mediated signals. It is important to understand how the chronic signal participates in a distinct molecular mechanism for mast cell degranulation.

**CONCLUDING REMARKS**

Cysteinyl LTs are known to be critical mediators of inflammation, and their roles in pathological inflammatory conditions such as asthma have been elucidated. The present study revealed a novel mode—an autocrine, constitutive mode—of cysteinyl LT production in mast cells. Furthermore, the present experiments conducted using BMMC showed that the autocrine secretion of constitutively produced cysteinyl LTs leads to chronic effects on mast cell degranulation, which is related to pro-allergic conditions. Targeted inhibition of CysLT1 has been established as an efficacious therapy for the treatment of these conditions. Since CysLT1 is expressed in a broad range of cell types, including epithelial cells, endothelial cells, smooth muscle cells, and most leukocytes, the net outcome of CysLT1 inhibition, i.e., the suppression of the functions of these cell types, is considered to be beneficial. Mast cells have been shown to potentially undergo a self-conditioning toward the onset of allergic symptoms. Hence, mast cells in the subclinical condition of allergic diseases are also important targets for CysLT1 inhibition during allergic therapy.

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