Leukotriene D₄ induces chemotaxis in human eosinophilic cell line, EoL-1 cells via CysLT1 receptor activation

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Abstract

Numerous reports have shown that cysteiny l leukotrienes (CysLTs) contribute to tissue accumulation of eosinophils in allergic airway inflammation. To date, only a few studies have reported that CysLTs promote chemotactic activity of human eosinophils in vitro. The purpose of this study was to investigate whether CysLTs promote chemotaxis in the human eosinophilic cell line, EoL-1. EoL-1 cells were induced to differentiate into mature eosinophil-like cells via incubation with butyric acid and cytokines (IL-3, IL-5 and GM-CSF). The chemotactic activity of the differentiated EoL-1 cells was assessed using the commercial cell migration assay kit. LTD₄ elicited dose-related chemotactic activity in the differentiated EoL-1 cells in the range of 1–100 nM. A typical bell-shaped dose-response curve was observed with optimal activity at 10 nM. The chemotactic activity elicited by LTD₄ (10 nM) was significantly inhibited by montelukast (control, 345 ± 19.2 × 10³ RFU; LTD₄ 10 nM alone, 511 ± 39.2 × 10³ RFU; LTD₄ 10 nM plus montelukast 100 nM, 387 ± 28.2 × 10³ RFU). LTD₄ induces migration in eosinophilic cells via activation of CysLT1 receptor. The present in vitro model may be useful for elucidation of the mechanism underlying CysLT-induced tissue eosinophilia.

Keywords: Pharmaceutical science, Immunology, Biological sciences
1. Introduction

The allergic response is a complex process involving the interaction of several mediators; among these, cysteinyl leukotrienes (CysLTs) represent one of the most important actors in the pathogenesis of airway allergic diseases such as allergic rhinitis and asthma [1]. Pharmacological studies using CysLTs indicate that two classes of receptor exist: CysLT1 receptor (CysLT1R) and CysLT2 receptor (CysLT2R) [1]. The involvement of CysLTs in eosinophil influx is an in vivo phenomenon that was first demonstrated in guinea pigs [2]. The inhalation of CysLTs results in a rise in sputum eosinophil counts within a few hours [3]. CysLT1R antagonists inhibit eosinophil recruitment during airway allergic inflammation [4], suggesting that CysLT1R may play important roles in allergic eosinophilic inflammation.

However, it is not clear whether CysLTs induce eosinophilic migration in vitro. Nagy et al. reported that neither leukotriene C₄ (LTC₄) nor leukotriene D₄ (LTD₄) caused chemotaxis of eosinophils [5]. However, Spada et al. reported contradictory findings, showing that CysLTs elicit chemotactic activity in eosinophils [6].

To date, studies of eosinophils have been hindered by the small number of cells that may be obtained from the peripheral blood of healthy donors, and the inability to expand eosinophils in vitro [7]. The human eosinophilic cell line EoL-1, represents a useful in vitro model for the study human eosinophils [8]. It has been demonstrated that EoL-1 cells may be induced to differentiate into eosinophilic granule-containing cells in response to a number of stimuli, such as treatment with butyric acid [9] and dibutyryl cyclic AMP (dbcAMP) [10].

In the present study, we aimed to clarify the effects of CysLTs and other lipid mediators on the the induction of the human eosinophilic EoL-1 cell line. Utilizing this eosinophilic leukemic cell line, we demonstrated that LTD₄ induces chemotaxis in EoL-1 cells via the activation of the CysLT1 receptor.

2. Methods

2.1. Cells and reagents

The human eosinophilic cell line, EoL-1, was purchased from the Riken Cell Bank (Tsukuba, Japan). RPMI 1640 culture medium, n-butyrate, IL-3, IL-5, and GM-CSF were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum was from Lonza Japan (Tokyo, Japan). LTC₄, LTD₄, leukotriene E₄ (LTE₄), and platelet activating factor (PAF) were from Cayman Chemical (Ann Arbor, MI, USA). The CysLT1 receptor antagonist, montelukast was from Sigma Chemical Co.
2.2. EoL-1 cell culture

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) in a humidified atmosphere at 37 °C with 5% CO2. Undifferentiated EoL-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum in 5% CO2 at 37 °C. Cells were induced to differentiate via the addition of n-butyrate (0.5 mM), IL-3 (1 ng/mL), IL-5 (1 ng/mL) and GM-CSF (0.5 ng/mL) for 5 days [9]. The culture medium was changed at day 1 and every 2 days thereafter. The cell concentration was adjusted to $1 \times 10^6$ cells/mL for chemotaxis assay.

2.3. Morphological analysis

For morphological analysis, the cells were allowed to dry on glass slides, and then processed with Hansel® stain (Torii Pharmaceutical Co. Ltd, Tokyo, Japan). Using the one minute technique per manufacturer instruction, the slides were first immersed with Hansel® stain and allowed to stand for 30 s. Distilled water was then added to take up the stain and the cells were allowed to stand for 30 s. The stain was then poured off and the slide was rinsed with distilled water to remove the excess stain. Slides were then quickly rinsed with 95% methyl alchol for fixation. The slides were observed using the Olympus BX51 microscope fitted with a DP70CCD camera (Olympus Optical, Tokyo, Japan).

2.4. Western blot analysis

For western blot analysis of CysLT receptors, rabbit anti-human CysLT1 receptor polyclonal antibody (1:1000; ab93481, Abcam, Cambridge, UK) and rabbit anti-human CysLT2 receptor polyclonal antibody (1:500; ab75160, Abcam) were used. EoL-1 cells were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), supplemented with a proteinase inhibitor cocktail (Sigma). The protein extracts were purified using the PAGEprep Advance Kit (Thermo Fisher Scientific Inc.). Protein concentration in the homogenates was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Protein extracts (10 μg protein each) were separated on a 4–12% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Invitrogen Corporation, Carlsbad, CA, USA). Membranes were then blocked with nonfat milk and probed with rabbit anti-human CysLT1 receptor or CysLT2 receptor polyclonal antibodies. The bands were visualized using a WesternBreeeze chromogenic western blot immunodetection kit (Invitrogen).
2.5. Chemotaxis assay

EoL-1 cell migration assay was performed using the CytoSelect™ Cell Migration Assay Kit (Cell Biolabs Inc., San Diego, CA). This kit contains polycarbonate membrane inserts (pore size; 5 μm) in a 24-well plate. The membrane serves as a barrier enabling the discrimination of migratory cells from non-migratory cells. Migratory cells extend protrusions towards chemoattractants and ultimately pass through the pores of the polycarbonate membrane. These migratory cells are then dissociated from the membrane and subsequently detected using CyQuant GR Fluorescent Dye.

The 24-well migration plate was allowed to warm up to room temperature under sterile conditions for 10 min. Cell suspension (1 × 10⁶ cells/mL) was prepared in serum-free RPMI 1640 media. DMSO (vehicle), and 10 nM or 100 nM montelukast sodium were added directly to the cell suspension 20 min prior to running the assay. Then, 0.5 mL of RPMI 1640 medium with 0.25% ethanol (vehicle), or chemoattractants was added to the lower well of migration plate. Next, 0.1 mL of the cell suspension solution was added the inside of each insert and incubated for 4–24 h in a cell culture incubator. After incubation, the media were carefully aspirated from the inside of the transwell insert. The insert were transferred to a clean well containing 225 μL of cell detachment solution and incubated for 30 min. The cells from the underside of the membrane were completely dislodged by gently tilting the insert several times in the detachment solution. Then, 75 μL of the 4 x Lysis Buffer/CyQuant GR Dye solution was added to each well containing cells along with 225 μL of cell detachment solution, and incubated for 20 min at room temperature. After incubation, 200 μL of the mixture was transferred to a 96-well plate suitable for fluorescence measurement. Fluorescence at 480 nm/520 nm was measured with a TriStar 2 LB942 fluorescence plate reader (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). The intensity of fluorescence was shown as relative fluorescence units (RFU).

2.6. Statistical analysis

Data are presented as means ± SD. Where appropriate, statistical differences were assessed by a non-parametric Mann-Whitney U test. P-values ≤0.05 were considered to indicate statistical significance.

3. Results

3.1. Differentiation causes morphological changes and up-regulation of CysLT receptors in EoL-1 cells

As shown in Fig. 1A, EoL-1 cells have the cytologicic characteristics of myeloblasts under the usual culture conditions (Fig. 1A-A). Cytokines (IL-3, IL-
5 and GM-CSF) (Fig. 1A-B), and n-butyrate (Fig. 1A-C) induced eosinophilic granule (+) cell differentiation. The stimulation with both cytokines and n-butyrate induced eosinophilic granule (+) cell differentiation on the most of EoL-1 cells (Fig. 1A-D). As shown in Fig. 1B, the ratio of eosinophilic granule (+) cells was the highest in both n-butyrate and cytokines treated cells (65.7 ± 11.5%).

In order to confirm the expression of CysLT receptors in undifferentiated and differentiated EoL-1 cells, western blot analysis was performed. As shown in Fig. 2A, the expression of a single band for the CysLT1 receptor and CysLT2 receptor proteins in EoL-1 cells was clearly demonstrated. The expression levels of
the CysLT receptors in the differentiated EoL-1 cells pretreated by the cytokines and n-butyrate were significantly higher than in undifferentiated EoL-1 cells (Fig. 2B).

3.2. Differentiation causes up-regulation of CysLTs-induced chemotaxis in EoL-1 cells

As a preliminary experiment, we examined the effects of LTC4, LTD4, and LTE4 on cell migration in undifferentiated and eosinophil-like differentiated EoL-1 cells. As shown in Fig. 3, CysLTs-induced migration was higher in differentiated cells than in undifferentiated cells. As LTD4 elicited the highest migration among the CysLTs (LTD4: 39% increase in comparison with the control), we focused on the chemotactic effects of LTD4 on differentiated EoL-1 cells. Fig. 4 shows the time kinetics of the migration of differentiated EoL-1 cells induced by LTD4 (1–100 nM) or control media. The difference in the number of migrating cells between the test
and control groups was highest at 12 h after stimulation. Accordingly, the dose-response of LTD₄-induced chemotaxis in differentiated EoL-1 cells at 12 h after the stimulation was assessed. As shown in Fig. 5, LTD₄ elicited a significant elevation in dose-related chemotactic activity in the range of 1–1,000 nM. Stimulation with 10 nM LTD₄ caused maximal chemotactic response (control, 335 ± 19.2 × 10³ RFU; LTD₄ 10 nM alone, 511 ± 39.2 × 10³ RFU).

Fig. 3. Differentiation caused upregulation of CysLTs-induced chemotaxis in EoL-1 cells. EoL-1 cell migration assay was performed using CytoSelect™ Cell Migration Assay kit, and the cell migration-inducing effects of CysLTs (LTC₄, LTD₄, and LTE₄) were evaluated using undifferentiated and differentiated (n-butyrat, IL-3, IL-5 and GM-CSF) EoL-1 cells. The chemotaxis assay was performed 12 h after the stimulation. Data are presented as means ± SD (n = 5). *P < 0.05, and **p < 0.01 by Mann-Whitney U test.

Fig. 4. Time kinetics of migration of differentiated EoL-1 cells induced by LTD₄ (1–100 nM) or control; the difference in number of migrating cells between the experiment group and the control was the highest at 12 h after stimulation. Data are presented as means ± SD (n = 5). *P<0.05, and **<0.01 by Mann-Whitney U test.
In order to clarify whether LTD₄ mediates signal transduction through the CysLT₁ receptor, the inhibitory effects of a CysLT₁ receptor antagonist on LTD₄-induced chemotaxis of differentiated EoL-1 cells were examined. As shown in Fig. 6, the CysLT₁ receptor antagonist montelukast (100 nM) significantly inhibited the 10 nM LTD₄-induced chemotaxis of differentiated EoL-1 cells. In contrast, montelukast did not affect the chemotaxis of differentiated EoL-1 cells elicited by PAF. Data are presented as means ± SD (n = 5).

**Fig. 5.** Induction of chemotaxis of differentiated EoL-1 cells by LTD₄. LTD₄ caused significantly elevated dose-related chemotactic activity in the range of 0.1–1,000 nM. Stimulation with 10 nM LTD₄ elicited maximal chemotactic response. Chemotaxis assay was performed 12 h after stimulation. Data are presented as means ± SD (n = 5). *P < 0.05, and **p < 0.01 by Mann-Whitney U test.

**Fig. 6.** Inhibitory effect of montelukast on LTD₄-induced chemotaxis. The CysLT₁ receptor antagonist montelukast (100 nM) significantly inhibited the 10 nM LTD₄-induced chemotaxis of differentiated EoL-1 cells. In contrast, montelukast did not affect the chemotaxis of differentiated EoL-1 cells elicited by PAF. Data are presented as means ± SD (n = 5).
nM LTD₄-induced chemotaxis of differentiated EoL-1 cells (control, 345 ± 19.2 × 10³ RFU; LTD₄ 10 nM alone, 511 ± 39.2 × 10³ RFU; LTD₄ 10 nM plus montelukast 10 nM, 484 ± 26.0 × 10³ RFU; LTD₄ 10 nM plus montelukast 100 nM, 387 ± 28.2 × 10³ RFU). In contrast, montelukast did not affect the chemotaxis of differentiated EoL-1 cells elicited by PAF. (PAF 100 nM alone, 644 ± 55.2 × 10³ RFU; PAF 100 nM plus montelukast 10 nM, 633 ± 46.3 × 10³ RFU).

4. Discussion

The purification of high numbers of circulating eosinophils is challenging as these cells account for less than 5% of peripheral blood leukocytes. Human eosinophilic leukemia cell line, EoL-1 cells differentiate into mature eosinophils when exposed to n-butyrate [11]. Therefore, the EoL-1 has been utilized to study the mechanisms underlying the development and function of eosinophils in eosinophilic allergic inflammation, e.g. during allergic rhinitis and asthma. Although IL-3, IL-5 and GM-CSF, do not themselves induce the differentiation of EoL-1 cells, the presence of these cytokines significantly augments n-butyrate-induced cell differentiation [9]. Therefore, in the present work, both n-butyrate and cytokines (IL-3, IL-5 and GM-CSF) were utilized to induce the differentiation of EoL-1 cells.

In the present study, the differentiation of EoL-1 cells induced by the addition of n-butyrate, IL-3, IL-5 and GM-CSF caused the acceleration of chemotactic activity in response to CysLTs; this was concomitant with the upregulation of the expression of CysLT1R and CysLT2R. Izumi et al. reported that the n-butyrate-induced differentiation of EoL-1 cells was associated with the induction of PAF receptor gene expression as well as the augmentation of PAF-induced increase in intracellular calcium concentrations [12]. However, no other study on the relationship between the chemotactic activity of EoL-1 cells in response to lipid mediators and cellular differentiation has been reported. It has been shown that the cell surface expression of CRTH2 in EoL-1 cells is significantly upregulated by INF-γ and TNF-α, and that PGD₂-induced EoL-1 chemotaxis is potentiated by these cytokines [13]. The present results and these previous reports suggest that the differentiation of EoL-1 cells into mature eosinophil-like cells leads to an increase in the expression of CysLT1 receptor and chemotactic activity in response to CysLTs.

In this study, cell migration in response to LTD₄ exhibited a typical bell-shaped dose-response curve with optimal activity at 10 nM. However, the effect of inhaled CysLTs on airway inflammatory cells in vivo has not been widely reported to date, therefore, the role of CysLTs in the development of allergen-induced airway eosinophilic inflammation remains poorly understood. Inhaled LTD₄ has been shown to increase sputum eosinophils in subjects with asthma in two out of four studies [14, 15, 16, 17]. Inhaled LTE₄ has been shown to elevate the number of eosinophils in sputum [17] and in bronchial biopsies [13].
workers reported that CysLTs significantly enhanced the chemotactic activity of eosinophils in the sputum of asthmatics, whereas sputum eosinophil chemotactic activity was significantly inhibited by montelukast in vitro [18]. Their report has been an important observation that made locally released-CysLTs potential contributors to eosinophilic inflammation. To date, there have been only a few reports of the chemotaxis-inducing effects of CysLTs on human eosinophils in vitro. Nagy and co-workers reported that LTD₄ did not elicit chemotaxis in eosinophils [5]. However, using an under-agarose technique, Spada and co-workers reported that LTD₄ acts as a potent chemoattractant for human eosinophils from healthy donors, with a significant effect at 0.1 nM [6]. This chemotactic activity was completely abolished by the CysLT1 receptor antagonist SKF 104353 [6]. Spada et al. hypothesized that the absence of CysLT-induced chemotactic activity in vitro was due to the use of eosinophils from subjects with hypereosinophilia [6]. However, Fregonese and co-workers reported that LTC₄, LTD₄, and LTE₄ may directly act as eosinophil chemoattractants in cells from atopic donors, and that eosinophil chemotaxis is completely abolished by the selective CysLT1 receptor antagonist montelukast [19]. From these contradictory reports, it is not clear whether CysLTs exert chemotactic effects on human eosinophils in vitro. Furthermore, there appear to be no other reports describing the chemotaxis-inducing effects of CysLTs on eosinophilic cell lines. Therefore, to our knowledge, the present findings in differentiated EoL-1 cells represent the first evidence for the induction of cell migration activity by CysLTs. Furthermore, the present in vitro model may be useful for clarifying the mechanisms underlying the induction of tissue eosinophilia by other lipid mediators.

In conclusion, we demonstrated that LTD₄ induces chemotaxis in EoL-1 cells via the activation of the CysLT1 receptor. This study provides novel insights into the role of CysLTs in airway eosinophilic inflammation. The present in vitro model may be useful for clarifying the mechanisms by which CysLTs induce tissue eosinophilia.

Declarations

Author contribution statement

Hideaki Shirasaki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Etsuko Kanaizumi, Tetsuo Himi: Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.
Funding statement

This work was supported by Grants-in-Aid for Scientific Research, Japan Society for the Promotion Science, Grant Number 15k10786.

Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2017.e00464

Acknowledgement

We would like to thank Editage (www.editage.jp) for English language editing.

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