Cysteinyl leukotrienes acting via granule membrane-expressed receptors elicit secretion from within cell-free human eosinophil granules

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Background: Cysteinyl leukotrienes (cysLTs) are recognized to act via receptors (cysLTRs) expressed on cell surface plasma membranes. Agents that block $cysLT_1$ receptor ($cysLT_1R$) are therapeutics for allergic disorders. Eosinophils contain multiple preformed proteins stored within their intracellular granules. Cell-free eosinophil granules are present extracellularly as intact membrane-bound organelles in sites associated with eosinophil infiltration, including asthma, rhinitis, and urticaria, but have unknown functional capabilities.

Objective: We evaluated the expression of cysLTRs on eosinophil granule membranes and their functional roles in eliciting protein secretion from within eosinophil granules.

Methods: We studied secretory responses of human eosinophil granules isolated by subcellular fractionation. Granules were stimulated with cysLTs, and eosinophil cationic protein and cytokines were measured in the supernatants. Receptor expression on granule membranes and eosinophils was evaluated by flow cytometry and Western blot.

Results: We report that receptors for cysLTs, $cysLT_1R$, $cysLT_2$ receptor, and the purinergic P2Y12 receptor, are expressed on eosinophil granule membranes. Leukotriene (LT) C_4 and extracellularly generated LTD_4 and LTE_4 stimulated isolated eosinophil granules to secrete eosinophil cationic protein. MRS 2395, a P2Y12 receptor antagonist, inhibited cysLT-induced eosinophil cationic protein release. Montelukast, likely not solely as an inhibitor of $cysLT_1R$, inhibited eosinophil cationic protein release elicited by LTC_4 and LTD_4 as well as by LTE_4.

Conclusion: These studies identify previously unrecognized sites of localization, the membranes of intracellular eosinophil granule organelles, and function for cysLT-responsive receptors that mediate cysteinyl leukotriene-stimulated secretion from within eosinophil granules, including those present extracellularly. (J Allergy Clin Immunol 2010;125:477-82.)

Key words: *Granules, cysteinyl leukotriene, eosinophil, allergy, asthma, montelukast*

Cysteinyl leukotrienes (cysLTs) constitute an important class of potent, proinflammatory mediators that are synthesized from membrane-derived arachidonic acid via the 5-lipoxygenase pathway leading to the formation of leukotriene (LT) A_4 that is converted into LTC_4 by the action of LTC_4 synthase.[1] Intracellular LTC_4 is actively transported extracellularly, where it is enzymatically converted sequentially to LTD_4 and then to LTE_4.[1] cysLTs are cell-membrane impermeant and are recognized to mediate their actions by engaging 2 heptahelical G protein–coupled receptors (GPCRs), designated $cysLT_1$ receptor ($cysLT_1R$) and $cysLT_2$ receptor ($cysLT_2R$), that are expressed on cell surface plasma membranes.[1,2] The rank order of affinities of cysLTs for human $cysLT_1R$ and $cysLT_2R$, based on transfected cells, is $LTD_4 > LTC_4 = LTE_4$ and $LTC_4 = LTD_4 > LTE_4$, respectively.[3,4]

Eosinophils, prominent leukocytes in allergic inflammation and anthelminthic responses,[5] are characterized by an abundance of intracellular granules that contain preformed proteins including distinct cationic proteins, such as eosinophil cationic protein (ECP), and a wide range of preformed cytokines, chemokines, and growth factors.[6,7] Human eosinophils are major sources of cysLTs and express both $cysLT_1R$ and $cysLT_2R$.[8] cysLTs and their receptors have critical roles in allergic diseases and represent important therapeutic targets for the control of asthma and other pathophysiological conditions.[9,10] Medications, including those recognized to inhibit ligand-binding to $cysLT_1R$, such as montelukast, are used in the management of asthma and related allergic diseases.[1]

In addition to its conventional plasma membrane expression, $cysLT_1R$ has been immunolocalized to nuclei in colorectal adenocarcinoma cells[11] and in a human mast cell line,[12] although the functions of nuclear $cysLT_1Rs$ have not been defined. We have recognized that some cytokine and chemokine receptors are richly present on eosinophil granules,[13] and we have recently demonstrated that eosinophil granules, on extrusion from eosinophils, respond to a stimulating cytokine, IFN-γ, and a chemokine, eotaxin-1 chemokine (C-C motif) ligand 11 (CCL11), via cognate granule membrane-expressed receptors, to activate intragranular signaling pathways that elicit granule protein secretion.[14] Intact membrane-bound eosinophil granules have long been recognized to be present extracellularly in tissues and secretions in many human eosinophil-enriched disorders, including asthma, rhinitis, urticaria, atopic dermatitis, eosinophilic esophagitis, and helminth infections.[15-21] The capacity of cell-free human eosinophil granules to act via receptor-mediated responses to polypeptide agonists and secrete granule-derived cytokines and cationic proteins has indicated that cell-free eosinophil granules may be functionally significant.[14] In the current study, we have evaluated whether receptors for cysLTs, in addition to their conventional

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Abbreviations used
cysLT: Cysteinyl leukotriene
cysLTR: Cysteinyl leukotriene receptor
$cysLT_1R$: Cysteinyl leukotriene 1 receptor
$cysLT_2R$: Cysteinyl leukotriene 2 receptor
ECP: Eosinophil cationic protein
GPCR: G protein–coupled receptor
LT: Leukotriene
P2Y12R: P2Y12 receptor

plasma and nuclear membrane localizations, are expressed and functional on the surface membranes of cell-free human eosinophil granules. We investigated the efficacy of intracellular LTC_4 and extracellular LTD_4 and LTE_4 as potential agonists of eosinophil granule secretion and the capacity of montelukast to inhibit cysLT-elicited eosinophil granule secretion.

METHODS

Eosinophil purification and subcellular fractionation

Eosinophils were purified from the blood of healthy and atopic donors as previously described.[14,22] Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Subcellular fractionation and eosinophil granule isolation were performed as described.[14,22] Briefly, eosinophils were disrupted by nitrogen cavitation (600 psi, 10 minutes) and postnuclear supernatants were ultracentrifuged (100,000*g*, 1 hour at 4°C) in linear isotonic Optiprep (Axis-Shield, Oslo, Norway) gradients (0% to 45%). Purity of isolated granules free of plasma membranes or other contaminating structures was rigorously ascertained as previously documented.[14,22]

Stimulation of isolated eosinophil granules

Subcellular fractions containing isolated granules were mixed with RPMI + 0.1% ovalbumin (without phenol red; Sigma, St Louis, Mo) followed by centrifugation (2500*g*, 10 minutes). Granule pellets were resuspended in 250 μL of the same medium. Treatments with montelukast (0.1 and 1 μmol/L; Merck, Rahway, NJ) and MRS 2395 (2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester, 1 and 10 μmol/L; Sigma), a selective P2Y12 receptor (P2Y12R) antagonist, were performed for 15 minutes before stimulation with LTC_4, LTD_4, or LTE_4 for 30 minutes at 37°C. After centrifugation at 4°C (2500*g*, 10 minutes), granule supernatants were collected and stored at –80°C. Drugs were diluted in dimethyl sulfoxide at a final concentration <0.01%, which had no effect on granule secretion.

Assays of granule-secreted proteins

The ECP levels in eosinophil granule supernatants were analyzed by a quantitative ECP ELISA kit (Medical & Biological Labs, Naka-ku Nagoya, Japan) according to the manufacturer's instructions. Stimulated ECP secretion represents ECP levels from stimulated samples minus ECP levels from unstimulated samples. Cytokines IL-4, IL-6, IFN-γ, IL-10, IL-12 (p70), IL-13, and TNF-α were quantified by using multiplex assays (Bio-Rad Laboratories, Inc, Hercules, Calif).

Flow cytometry of isolated granules and eosinophils

Isolated granules or eosinophils were incubated for 1 hour with primary antibody or primary antibody premixed with blocking peptide. Then the granules were washed and incubated with the respective fluorescein isothiocyanate–conjugated secondary antibodies for 15 minutes on ice in the absence of granule fixation. After staining, granules were fixed in buffer containing 2% paraformaldehyde without methanol (Electron Microscopy Sciences, Fort Washington, Pa) for 5 minutes. Control or nonimmune antibodies were included for all. Analyses were performed on a FACScan with CELLQUEST software (BD Biosciences, San Jose, Calif).

Mouse antihuman P2Y12R polyclonal antibody (1:100) was purchased from Abnova Corp (Taipei, Taiwan). Goat polyclonal antibody against a peptide mapping the N-terminus domain of $cysLT_1R$ (N-20; 5 μg/mL) and the blocking peptide (20 μg/mL) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Rabbit polyclonal antibodies against a peptide mapping the C-terminus of the $cysLT_1R$ (5 μg/mL) and the N-terminus of the $cysLT_2R$ (5 μg/mL) and the blocking peptide (20 μg/mL) were purchased from Cayman Chemical (Ann Arbor, Mich). Fluorescein isothiocyanate–conjugated F(ab')2 goat antimouse, donkey antigoat, and goat antirabbit IgGs were used as secondary antibodies (1:100). Mouse, goat, and rabbit normal IgGs were used as control antibodies (Jackson Immuno Research Inc, West Grove, Pa).

Western blotting

Granules and eosinophils were lysed in lithium dodecyl sulfate (LDS) sample reducing buffer (Nupage; Invitrogen, Carlsbad, Calif) and boiled for 5 minutes. Samples were loaded on 10% Bis-Tris gels (Invitrogen) and run using 3-(n-morpholino) propanesulfonic acid (MOPS) running buffer. Gels were transferred to nitrocellulose membranes (Thermo Fisher Scientific, San Jose, Calif), blocked with 5% milk for at least 1 hour, and probed with rabbit anti-P2Y12R polyclonal antibody (1:400; Alomone Labs, Jerusalem, Israel) or the antibody premixed with the blocking peptide overnight. Antirabbit antibody conjugated to horseradish peroxidase (1:15,000; Jackson Immuno Research Inc) was used as secondary Ab. Membranes were developed with West Femto chemiluminescence kits (Thermo Fisher Scientific).

Statistical analysis

Secreted ECP levels, means of duplicates ± SDs, are ECP levels from stimulated granules minus ECP levels from unstimulated granules. Data are shown for 1 experiment representative of 3. Quantities of unstimulated and stimulated ECP secreted varied among replicate experiments, but the patterns of release and statistical differences were consistent in each of the replicate experiments. Results were analyzed by 1-way ANOVA, followed by the Newman-Keuls test. P values <.05 were considered significant (2-tailed test).

RESULTS

Extracellular eosinophil granules express on their membranes amino-terminal, ligand-binding domains for cysLT receptors

To evaluate whether secretory responses of eosinophil granules, as intracellularly resident or extracellularly released organelles, might be mediated by intracrine or paracrine acting cysLTs, we first analyzed by flow cytometry the expression of $cysLT_1R$ and $cysLT_2R$ proteins on the surface membranes of isolated human eosinophil granules. Without membrane permeabilization, granules displayed immunoreactivity for both $cysLT_1R$ (Fig 1, *A*) and $cysLT_2R$ (Fig 1, *B*) using polyclonal antibodies raised against epitopes present specifically in the nominally "extracellular," ligand-binding regions of each cysLT receptor (cysLTR). Specificities of each polyclonal antibody for $cysLT_1R$ (Fig 1, *A*) and $cysLT_2R$ (Fig 1, *B*) were corroborated by complete neutralization of immunostaining through preincubation of the anti-$cysLT_1R$ and anti-$cysLT_2R$ polyclonal antibodies with their respective specific blocking peptide immunogens. In contrast, eosinophil granules exhibited no staining with a polyclonal