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Research paper

Leukotriene receptor expression in mast cells is affected by their agonists

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ABSTRACT

The effects of LTs are mediated by GPCRs: cysLTs interact with CYSLTR1, CYSLTR2, or GPR17, and LTB_4 acts *via* BLT1R or BLT2R. Data relating to the presence of these receptors in mature tissue mast cells are not entirely known. By confocal microscopy with image analyses and flow cytometry, we established that native rat mast cells isolated from peritoneal cavity constitutively express all studied receptors. Moreover, we clearly documented that LTs by themselves can influence their own receptor expression. Low concentrations of LTs induce translocation of LT receptors from cell interior to plasma membrane, which can lead to increased mast cell responsiveness to LT stimulation. High concentrations of LTs cause internalization and, in consequence, reduction in the number of receptors on the cell surface, and it may result in desensitization of mast cells to subsequent LT stimulation. These observations may imply a physiological feedback mechanism regulating mast cell sensitivity to LT activation within tissues.

1. Introduction

Leukotrienes (LTs), both LTB_4 and cysLTs, namely LTC_4, LTD_4, and LTE_4, are the family of lipid mediators derived from lipoxygenase pathway of the arachidonic acid (AA) metabolism. The production of LTs requires the reversible translocation of 5-lipoxygenase (5-LO) from the nucleoplasm or the cytosol to the perinuclear region. This enzyme, acting in concert with 5-lipoxygenase-activating peptide (FLAP), converts AA into 5 hydroperoxyeicosatetraenoic acid (5-HPETE), and next into LTA_4. The unstable LTA_4 can be converted into either LTB_4 or LTC_4, and the latter is metabolized to LTD_4 and LTE_4 [1–4]. LTs are synthesized by cells of the innate immune system in response to different immune and inflammatory stimuli. CysLTs are generated by various types of leukocytes, including eosinophils, basophils, mast cells, and macrophages, while LTB_4 is produced mainly by neutrophils, monocytes, and mast cells. It is noteworthy that LTs can also be produced through transcellular biosynthesis. Such transcellular pathway of LT synthesis has been described for macrophages, mast cells, monocytes, airway epithelial cells, kidney-derived endothelial cells, chondrocytes, and keratinocytes [5,6]. It has been well established that LTs exert pleiotropic effects and play an important role in the development of inflammation, as they stimulate different cells to the production of proinflammatory mediators and cytokines, promote cell adhesion to the vascular epithelium, and have the potency to recruit proinflammatory cells to the site of inflammation [1,2,4].

The biological effects of LTs are mediated by GPCRs. The cysLTs interact with specific cysLT receptors, namely CYSLTR1, CYSLTR2, and GPR17 [1,2,7]. Recent findings imply there is one more cysLT receptor specific for LTE_4, namely GPR99 [8,9]. LTB_4 acts through two receptors, BLT1R and BLT2R, with high- and low-affinity to the ligand, respectively [3]. It should be stressed that cellular distribution of LT receptors is varied. CYSLTR1 is widely expressed by peripheral leukocytes, monocytes, macrophages, Th2 lymphocytes, B cells, but is also present in various types of structural cells. In addition to granulocytes and macrophages, expression of CYSLTR2 was found in cardiac Purkinje fiber cells, cardiac and vascular smooth muscles, endothelium and epithelium, adrenal chromaffin cells or brain cells [1,2]. The GPR17 is primarily expressed in the central nervous system, in particular on oligodendrocyte precursor cells, and in the kidney and heart [7]. The BLT1R expression has been reported predominantly in activated leukocytes, while BLT2R seems to be more ubiquitous, with the highest level in lymphocytes and hepatocytes [3].

Mast cells are long-lived resident connective tissue cells distributed throughout the body, and are especially numerous beneath the subepithelial layers of the skin, in the respiratory system, in the gastrointestinal and genitourinary tracts, and adjacent to blood vessels and nerves. These cells are a potent source of diverse mediators such as cytoplasmic granule-associated preformed mediators (e.g. histamine, proteases, proteoglycans, metalloproteinases), *de novo* generated and secreted arachidonic acid metabolites (i.e. LTs, prostaglandins (PGs),

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thromboxanes) as well as a lot of cytokines and chemokines [10,11]. Mast cell-derived products can exert diverse pro-inflammatory and anti-inflammatory effects, and influence tissue turnover. That is why, these cells take part not only in homeostasis maintenance but also play an important role in various physiological processes, tissue remodeling and repair, and host defense against pathogens. Mast cells strongly influence both innate and acquired immune responses and are involved in the development of many pathological conditions, including allergic reactions [11–13]. Undoubtedly, they play a crucial role in both acute and chronic inflammation, as well [14,15].

Considering the meaningful role of mast cells in physiological and pathological processes is it important to comprehend endogenous factors affecting their biology and activity. It was documented that mast cells constitutively express receptors for cytokines and chemokines, neuropeptides and hormones, certain complement products, different cell-derived peptides and proteases, PGE_2, or histamine [10,11,16–18]. So far, data relating to the presence of receptors for cysLTs and/or LTB_4 on mature mast cells, and consequently LT significance in modulation mast cell activity within the tissue, are not entirely known. Therefore, the aim of our study was to determine the expression of receptors for cysLTs and LTB_4 in fully mature connective tissue mast cells. We also scheduled to establish whether LTs by themselves affect the expression of their own receptor in mature mast cells.

2. Materials and methods

2.1. Reagents

DMEM, HBSS, sodium bicarbonate, FCS, gentamicin, and glutamine were purchased from GIBCO (Gaithersburg, MD). 10% Tris-glycine-SDS precast gel and Tris-glycine-SDS sample buffer were purchased from Invitrogen (Gaithersburg, MD). Percoll, toluidine blue, trypan blue, PBS, RIPA buffer, and saponin were obtained from Sigma-Aldrich (St. Louis, MO). LTB_4, LTC_4, LTD_4, LTE_4, the FLAP inhibitor MK886, CYSLTR1 blocking peptide, CYSLTR2 blocking peptide, GPR17 blocking peptide, BLT1R blocking peptide, BLT2R blocking peptide were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against CYSLTR1, CYSLTR2, GPR17, BLT1R, and BLT2R, as well as anti-phospho-p38 (Tyr-182), anti-p38, anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-ERK1/2, anti-β-actin, isotype control antibodies, HRP-labeled antibodies, and fluorescein isothiocyanate (FITC)-conjugated polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). CellFIX was purchased from BD Biosciences (Benelux, NV). RNeasy Mini Kit was obtained from Qiagen (Valencia, CA). The High Capacity cDNA Reverse Transcription Kit, TaqMan® probes dyed FAM, rCYSLTR1 (Rn00586294_s1), rCYSLTR2 (Rn00824657_s1), rGPR17 (Rn03020713_s1), rBLT1R (Rn00572209_s1), rBLT2R (Rn00586281_s1), rIL-1β (Rn00580432_m1), rCCL2 (Rn00580555_m1), rCCL3 (Rn01464736_g1), rGM-CSF (Rn01456850_m1), rIL-33 (Rn01759835_m1), rCXCL8 (Rn00578225_m1), rActb (Rn00667869_m1), and TaqMan® Gene Expression Master Mix were purchased from Applied Biosystems (Foster City, CA). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore (Bedford, MA). The Western Lightning luminol-based enhanced chemiluminescence (ECL) Pro system was purchased from Perkin-Elmer (Boston, MA) and bicinchoninic acid (BCA) Protein Assay Kit was obtained from Pierce (Rockford, IL).

2.2. Mast cell isolation

Mast cells were collected from peritoneal cavities of female albino Wistar rats and purified by a density gradient separation method, as previously described [19,20]. After isolation mast cells were counted and resuspended in an appropriate volume of complete (c)DMEM to obtain a concentration of 1.5×10^6 cells/ml. Mast cells were prepared with purity > 98%, as determined by metachromatic staining with toluidine blue. The viability of mast cells was over 98%, as estimated by trypan blue exclusion assay. Animal experiments were approved by the Local Ethics Committee for Experiments on Animals of the Medical University of Lodz (the approval number 44ŁB 673/2013).

2.3. Quantitative RT-PCR

qRT-PCR was used to determine CYSLTR1, CYSLTR2, GPR17, BLT1R, and BLT2R mRNA levels. Purified mast cells suspended in cDMEM were pretreated with MK886 (100 nM, 30 min, 37 °C) and next stimulated with LTB_4, LTC_4, LTD_4, or LTE_4 at final concentrations of 0.1 and 100 nM in a humidified atmosphere with 5% CO_2 for 45 min at 37 °C. MK886 binds to FLAP with high affinity and inhibits endogenous LT biosynthesis. The same technique was used to estimate cytokine/chemokine transcripts in MK886-pretreated cells stimulated with 100 nM LTs for 2 h. For control, mast cells were incubated under the same conditions without LTs. qRT-PCR was conducted as previously described [19,20]. The mRNAs expression was corrected by normalization based on the transcript level of the housekeeping gene rat Actb.

2.4. Confocal microscopy

For determination of constitutive and LT-induced cellular distribution of CYSLTR1, CYSLTR2, GPR17, BLT1R, and BLT2R protein confocal microscopy was used. To determine the subcellular localization of LT receptors mast cells were permeabilized with 0.1% saponin for 30 min at room temperature. Constitutive expression of LT receptors was assessed in non-stimulated cells. LT-induced receptor expression was estimated in MK886-pretreated mast cells incubated with LTB_4, LTC_4, LTD_4, or LTE_4, at final concentrations of 0.1 and 100 nM for 45 min in a humidified atmosphere with 5% CO_2 at 37 °C. For comparison, receptor expression was also determinate in permeabilized cells incubated with MK886 under the same conditions. After that mast cells were fixed with CellFIX solution for 15 min and washed twice with 1% PBS. Permeabilized mast cells were stained for 1 h with appropriate antibodies (antibody dilution 1:100). Next, cells were washed with 1% PBS and incubated for 1 h with FITC-conjugated secondary antibodies in 1% PBS. Finally, cells were washed and resuspended in 1% PBS. For confocal microscopy, the samples were mounted on microscope slides. Images were captured using a confocal laser scanning microscope (LSM510 Meta, Zeiss) combined with an Axiovert 200 M (Zeiss) inverted microscope equipped with a Plan-Neofluar objective (40x/0.6). All settings were held constant throughout the experiments except for gain factor which was adjusted for each receptor. The fluorescence was recorded using the argon laser (488 nm) and a BP filter set (505 nm). The same laser line was used for Nomarski DIC. All signals obtained from confocal microscopy were validated with profile view image analysis and the diagrams presenting intensity values are placed below each microphotograph.

2.5. Flow cytometric analysis (FACS)

For determination of constitutive and LT-induced cell surface CYSLTR1, CYSLTR2, GPR17, BLT1R, and BLT2R protein expression flow cytometry technique was used. Constitutive expression of LT receptors was assessed on freshly isolated native mast cells (non-stimulated cells). LT-induced receptor expression was estimated on mast cells pretreated with MK886 and next incubated with LTB_4, LTC_4, LTD_4, or LTE_4, at final concentrations of 0.1 and 100 nM for 45 min in a humidified atmosphere with 5% CO_2 at 37 °C. For control, receptor expression was determined on mast cells preincubated with MK886 or medium alone under the same conditions. After that mast cells were fixed with CellFIX solution for 15 min and washed twice with 1% PBS. Next, mast cells were resuspended in 1% PBS and stained for 1 h with appropriate antibodies (dilution 1:100). For control, mast cells were