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Neutralization of leukotriene C_4 and D_4 activity by monoclonal and single-chain antibodies

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ABSTRACT

Background: Cysteinyl leukotrienes (LTs) are key mediators in inflammation. To explore the structure of the antigen-recognition site of a monoclonal antibody against LTC_4 (mAbLTC), we previously isolated full-length cDNAs for heavy and light chains of the antibody and prepared a single-chain antibody comprising variable regions of these two chains (scFvLTC).
Methods: We examined whether mAbLTC and scFvLTC neutralized the biological activities of LTC_4 and LTD_4 by competing their binding to their receptors.
Results: mAbLTC and scFvLTC inhibited their binding of LTC_4 or LTD_4 to $CysLT_1$ receptor ($CysLT_1R$) and $CysLT_2$ receptor ($CysLT_2R$) overexpressed in Chinese hamster ovary cells. The induction by LTD_4 of monocyte chemoattractant protein-1 and interleukin-8 mRNAs in human monocytic leukemia THP-1 cells expressing $CysLT_1R$ was dose-dependently suppressed not only by mAbLTC but also by scFvLTC. LTC_4- and LTD_4-induced aggregation of mouse platelets expressing $CysLT_2R$ was dose-dependently suppressed by either mAbLTC or scFvLTC. Administration of mAbLTC reduced pulmonary eosinophil infiltration and goblet cell hyperplasia observed in a murine model of asthma. Furthermore, mAbLTC bound to $CysLT_2R$ antagonists but not to $CysLT_1R$ antagonists.
Conclusions: These results indicate that mAbLTC and scFvLTC neutralize the biological activities of LTs by competing their binding to $CysLT_1R$ and $CysLT_2R$. Furthermore, the binding of cysteinyl LT receptor antagonists to mAbLTC suggests the structural resemblance of the LT-recognition site of the antibody to that of these receptors.
General significance: mAbLTC can be used in the treatment of inflammatory diseases such as asthma.

1. Introduction

Cysteinyl leukotrienes (LTs) including LTC_4, LTD_4 and LTE_4 are key mediators in inflammation [1,2]. Biosynthesis of cysteinyl LTs is initiated by conversion of arachidonic acid to LTA_4 catalyzed by 5-lipoxygenase. Then, the unstable epoxide LTA_4 is conjugated with reduced glutathione to produce LTC_4 which is further converted to LTD_4 and LTE_4 by the sequential cleavage for the tripeptide adduct of glutamate and glycine [2]. The biological activities of the cysteinyl LTs are mediated by at least two known G protein-coupled receptors, $CysLT_1$ receptor ($CysLT_1R$) and $CysLT_2$ receptor ($CysLT_2R$) [3]. The human and mouse $CysLT_1Rs$ bind LTD_4 with higher affinity than LTC_4 [4,5]. The human $CysLT_2R$ binds LTC_4 and LTD_4 equally [6], whereas the mouse $CysLT_2R$ shows higher affinity to LTC_4 than to LTD_4 [5]. $CysLT_1R$ couples to $G_{q/11}$ and/or $G_{i/o}$ depending on cell types, whereas $CysLT_2R$ couples to $G_{q/11}$ and activation of these receptors elicits intracellular calcium mobilization [3]. $CysLT_1R$ is the molecular target of the antiasthmatic drugs pranlukast, zafirlukast and montelukast, which show efficacy in blocking inflammatory actions in the airways and improving airway function [7–9]. The functional characterization of $CysLT_2R$ had been limited, although its roles in bleomycin-induced pulmonary fibrosis [10] as well as atopic dermatitis [11] were recently reported. BAY-u9773 has been known as a

Abbreviations: BAL, bronchoalveolar lavage; CHO, Chinese hamster ovary; $CysLT_1R$, $CysLT_1$ receptor; $CysLT_2R$, $CysLT_2$ receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; IL-8, interleukin-8; LT, leukotriene; mAbLTC, monoclonal antibody against leukotriene C_4; MCP-1, monocyte chemoattractant protein-1; OVA, ovalbumin; PAS, periodic acid-Schiff; scFvLTC, single-chain variable fragment comprising variable regions of heavy and light chains of monoclonal antibody against leukotriene C_4

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nonselective antagonist for $CysLT_2R$, but two selective $CysLT_2R$ antagonists, HAMI3379 and $BayCysLT_2$ were recently developed [12,13].

Matsumoto et al. previously prepared a monoclonal antibody against LTC_4 (mAbLTC) for immunoaffinity purification and radioimmunoassay of this bioactive eicosanoid in human synovial fluid [14]. To explore the structure of the active site of the antibody recognizing the antigen, we isolated full-length cDNAs for heavy and light chains of the monoclonal antibody and deduced their primary structures [15]. Furthermore, we constructed an expression plasmid encoding a single-chain variable fragment antibody comprising variable regions of heavy and light chains of the original monoclonal antibody and expressed it in COS-7 cells [15]. The expressed fragment termed as scFvLTC showed high affinity and specificity to LTC_4 but lower affinity to LTD_4 and LTE_4, although a slight reduction of affinity to these LTs was observed as compared with mAbLTC [15]. The aim of this study was to examine whether mAbLTC and scFvLTC neutralized the biological activities of LTC_4 and LTD_4 by competing their binding to their receptors. Furthermore, we examined whether mAbLTC bound to antagonists for these receptors and investigated the structural similarity of the LT-recognition site of antibody to that of receptors.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice were obtained from Charles River Laboratories, Japan (Yokohama, Japan), and were used under protocols that were approved by the Experimental Animal Care and Use Committee of Okayama Prefectural University. All mice were maintained in a temperature-controlled (25 °C) facility with a 12-hour light/12-hour dark cycle and were fed a normal rodent chow diet (CE-2, CLEA Japan, Tokyo, Japan). Food and water were available ad libitum.

2.2. Materials

LTC_4, LTD_4, pranlukast, MK-571, BAY-u9773, HAMI3379, $BayCysLT_2$, goat anti-mouse IgG-coated plates and Ellman's reagent containing the substrate to acetylcholinesterase were purchased from Cayman Chemical (Ann Arbor, MI). LTC_4-acetylcholinesterase conjugate was a gift from Dr. K. Maxey of Cayman Chemical. Oligodeoxyribonucleotides were synthesized by Hokkaido System Science (Sapporo, Japan). An expression vector pCXN2 having a powerful CAG promoter [16] was kindly provided by Dr. J. Miyazaki of Osaka University. Hybridoma cells producing mAbLTC [14] were cultured in serum-free GIT medium (Wako, Osaka, Japan). The mAbLTC in the culture medium was purified using protein A-Sepharose CL4B (GE Healthcare, Piscataway, NJ) column chromatography and the buffer was changed to phosphate-buffered saline at pH 7.4 by gel-filtration before use [15].

2.3. Preparation of scFvLTC

The scFvLTC was prepared as described previously [15]. Briefly, an expression plasmid encoding an scFvLTC (pCXN2-scFvLTC) [15] was introduced into COS-7 cells by the DEAE-dextran method. The culture medium was collected and subjected to Ni-NTA agarose (Qiagen, Valencia, CA) column chromatography. After washing with 50 mM sodium phosphate buffer at pH 8.0 containing 300 mM NaCl, 0.05% Tween 20 and 20 mM imidazole, the hexahistidine-tagged scFvLTC was eluted with 250 mM imidazole in 50 mM sodium phosphate buffer at pH 8.0 containing 300 mM NaCl and 5 mM NaN_3. After desalting and concentration, the eluted fractions were reloaded onto a Ni-NTA agarose. The purified scFvLTC was obtained as described above except that 50 mM imidazole was contained in the washing buffer, and the buffer was changed to phosphate-buffered saline at pH7.4 before use.

2.4. Cell culture

Chinese hamster ovary (CHO) cells were cultured at 37 °C with 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Human monocytic leukemia THP-1 cells obtained from RIKEN Cell Bank (Tsukuba, Japan) were maintained at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum. Mouse macrophage-like J774A.1 cells obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) were maintained at 37 °C with 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum. The cells were subcultured every 3–4 days.

2.5. Transfection of human $CysLT_1R$ and $CysLT_2R$ expression vectors

cDNAs of human $CysLT_1R$ and $CysLT_2R$ were isolated from human leukocyte cDNA library [5], and ligated to EcoRI site of pCXN2 expression vector. CHO cells were transfected with the plasmid using lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 3 days, the transfected cells were split at a ratio of 1: 100 in culture medium containing 1.5 mg/ml geneticin. After 2 weeks we picked clones, and identified receptor-expressing cells by dot-blot analysis of RNA with alkaline phosphatase-labeled cDNA probes corresponding to the full-length coding sequences of human $CysLT_1R$ and $CysLT_2R$ using AlkPhos Direct Labelling Module (GE Healthcare). The cells permanently expressing human $CysLT_1R$ and $CysLT_2R$ were designated as CHO-$CysLT_1$ and CHO-$CysLT_2$, respectively. Mock-transfected cells were established by transfection of the parental pCXN2.

2.6. Measurement of intracellular calcium concentrations

CHO-$CysLT_1$, CHO-$CysLT_2$ and THP-1 cells were suspended in Hepes-Tyrode's-BSA buffer (10 mM Hepes-NaOH at pH 7.4, 140 mM NaCl, 2.7 mM KCl, 0.49 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4 and 0.1% (w/v) of fatty acid-free BSA) and loaded with 5 μM fura 2-AM at 37 C for 1 h, washed twice and resuspended in Hepes-Tyrode's-BSA buffer to a concentration of 2×10^6 CHO cells/ml and 5×10^6 THP-1 cells/ml. Receptor antagonists or antibodies were added. After stirring at 37 C for 5 min, LTC_4 or LTD_4 was added, and fluorescence was measured using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan), with emission wavelength set at 510 nm and excitation wavelength at 340 nm and 380 nm. The intracellular calcium concentration was calculated with the Grynkiewicz formula [intracellular calcium concentration $= K_d \times (F_0/F_s) \times [(R - R_{min}) / (R_{max} - R)]$]. In this formula R is the experimentally derived 340/380 nm fluorescence ratio, R_{min} and R_{max} are the values of ratio pairs in the presence of nominally zero and saturating calcium, respectively. K_d is the fura-2 dissociation constant (224 nM at 37 C), and F_0 and F_s are proportionality coefficients for free- and calcium-bound fura-2, respectively (measured at 380 nm excitation). R_{max} was measured in the buffer containing 10 μM ionomycin and 20 mM $CaCl_2$ and R_{min} in the buffer containing 10 μM ionomycin and 10 mM EGTA.

2.7. Quantitative RT-PCR analysis

THP-1 cells were seeded into 35 mm-dishes at a density of 1×10^5 cells/dish in RPMI 1640 and preincubated at 37 C for 24 h. Receptor antagonists or antibodies were added 5 min before stimulation of the cells. After addition of LTC_4 or LTD_4, the cells were incubated for 1 h. Total RNA was isolated from the cells using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and oligo$(dT)_{20}$ as a primer. The quantitative RT-PCR analyses were performed using a Bio-Rad iQ5 real time PCR detection system (Hercules, CA). Monocyte chemoattractant protein-1 (MCP-1)-specific primers were: upstream, 5′-GCTCAGCCAGATGCAATCAA-3′ and downstream,