이학 석사학위 논문

Effect of Cysteinyl Leukotriene D4 on Chemokine Expression via Cysteinyl Leukotriene Receptor 1 in Human Lung Epithelial Cells, A549

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의 학 과

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by

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김설화의 이학 석사학위 논문을 인준함.

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-ABSTRACT-

Effect of Cysteinyl Leukotriene D4 on Chemokine Expression via Cysteinyl Leukotriene Receptor 1 in Human Lung Epithelial Cells, A549

Background : Cysteinyl leukotrienes (CysLTs) such as LTC4, LTD4 and LTE4 are proinflammtory lipid mediators synthesized by the 5-lipoxygenase pathway from arachidonic acid and play important roles in eosinophilic chemotactic activity to airway inflammation characterized by bronchoconstriction, mucus secretion and airway hyperresponsiveness through G-protein coupled receptor, cysteinyl leukotriene receptor 1(CysLTR1). CCL2 (MCP-1, Monocyte Chemoattractant protein-1) is one of the major chemokines responsible for attracting monocytes. CXCL10 (IP-10, IFN-γ-inducible protein 10kDa) is an IFN-γ-inducible chemokine that preferentially attracts activated Th1 lymphocytes.

Objective: The aim of this study is to investigate transcriptional regulation of MCP-1 and IP-10 by LTD4 stimulation via CysLTR1 in human lung epithelial A549 cells.

Method: To investigate the effect of LTD4 and CysLTR1 on MCP-1 and IP-10, Two different kinds of epithelial cells, A549 cells and CysLTR1 over-expressed A549 cells, were prepared and treated with LTD4 100nM for 0.5, 1, 2, 4 and 8hours. After LDT4 stimulation, mRNA levels of MCP-1 and IP-10 were examined by real-time

RT-PCR. To make sure whether MCP-1 and IP-10 are involved with CysLTR1, prior to treatment of LTD4, cells were pre-treated with CysLTR1 antagonist, MK-571 100nM for 3hrs. To confirm the effect of LTD4 and/or CysLTR1 on releasing MCP-1 and IP-10, ELISA was performed with cell supernatant.

Result: mRNA expression level of MCP-1 was augmented as soon as treated with LTD4. mRNA expression level of IP-10 was increased 1hr after LTD4 100nM stimulation in A549cells. After transfection of CysLTR1, the mRNA expression of both MCP-1 and IP-10 were enhanced about more than 500-fold and 300-fold respectively. In case of released protein, IP-10 were increased about more than 100 times whereas MCP-1 was not affected. Although the tendency of the effect of LTD4 on IP-10 after transfection of CysLTR1 in mRNA level was similar with one before transfection, protein level of IP-10 was increased preferentially at 1hr after LTD4 treatment in CysLTR1-transfected A549 cells. MK571, CysLTR1 antagonist inhibited the expression of IP-10 in mRNA level while couldn't block MCP-1 expression. IP-10 blocked by MK-571 was recovered immediately by LTD4 treatment.

Conclusion : IP-10 and MCP-1 are up-regulated by LTD4 stimulus both in transcriptional and translational levels. Up-regulation of IP-10 by LTD4 was specifically inhibited by MK-571, cysLTR1 specific antagonist while the increased expression of MCP-1 was not. These data suggest that IP-10 expression may be involved in the pathogenic mechanism of asthma via CysLTR1.

Key words : asthma, cysteinyl leukotriene D4 (LTD4), cysteinyl leukotriene receptor 1 (CysLTR1), cytokines, chemokines, MCP-1(CCL2), IP-10(CXCL10), MK-571

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ABBREVIATION

LTD4 : cysteinyl leukotriene D4

CysLTR1 : Cysteinyl leukotriene receptor type I

MCP-1 : Monocyte Chemoattractant Protein – 1 (CCL2)

IP-10 : Interferon-ɣ-inducible Protein 10kDa (CXCL10)

CCL4 : Macrophage inflammatory protein 1-beta, Lymphocyte activation gene 1 protein

CCL8 : Monocyte chemoattractant protein 2

CCL 16 : small inducible cytokine subfamily A, member 16

CCL 23 : Macrophage inflammatory protein 3

CCL25 : Thymus expressed chemokine, Ck beta-15

CCR5 : chemokine (C-C motif) receptor 5

CCR8 : C-C chemokine receptor type 8

CRP : C-reactive protein precursor

CXCL6 : granulocyte chemotactic protein 2

CXCL11 : Interferon-inducible T-cell alpha chemoattractant

CXCL12 : Pre-B cell growth-stimulating factor

CXCL13 : B lymphocyte chemoattractant

CXCL14 : Small inducible cytokine B14 precursor

ICEBERG : Caspase-1 inhibitor Iceberg

IL1F6 : interleukin 1 family, member 6 (epsilon)

IL13 : Interleukin-13 precursor

LTA : Lymphotoxin-alpha precursor, Tumor necrosis factor ligand superfamily member 1

Ⅰ. INTRODUCTION

Asthma is known as a chronic inflammatory disease caused by infiltration of inflammatory cells such as T cells, eosinophils, mast cells. In the pathogenesis of asthma, leukotrienes (LTs) play an important role by inducing bronchoconstriction, vascular hyperpermeability, mucus hypersecretion, airway hypersensitivity, and eosinophil recruitment (Kanaoka et al., 2004). They are metabolic products of arachidonic acid by cytosolic phospholipase A2 (cPLA2) from phospholipids in cellular membranes (Bandeira-Melo et al., 2003, Henderson, 1994, Wenzel, 2003). Especially, cysteinyl leukotrienes (CysLTs, known to consist of LTC4, and its conversion products, LTD4 and LTE4) are generated via 5-lipoxygenase pathway of arachidonic acid metabolism in a number of inflammatory cells including eosinophils, basophils, monocytes, and macrophages (Lewis et al., 1990, O'Byrne, 1997).

CysLTs affect the inflammatory response by inducing the production of cytokines required to stimulate T-cell responses or determine the homing of dendritic cells, and attract leukocytes to initiate inflammatory responses either directly or indirectly (Kanaoka et al., 2004). Many studies have reported that high level of cysLTs were detected in urine and/or exhaled air condensate of asthmatic patients (Antczak et al., 2002, Capra et al., 2006, Green et al., 2004, Montuschi et al., 2002, O'Byrne, 1997, Sampson et al., 1995). In addition, inhalation of LTD4 induced bronchoconstriction and sputum eosinophilia in patients with asthma, while inhaled methacholine, other bronchoconstrictor, didn’t affect eosinophil counts

(Diamant et al., 1997, Fregonese et al., 2002).

The bronchoconstrictive effect of cysLTs is exerted via two types of their G protein-coupled receptor, cysteinyl leukotriene receptor type 1 (CysLTR1) and type 2 (CysLTR2) (Brink et al., 2003, Capra et al., 2004). In particular, CysLTR1 is expressed in airway smooth muscle cells, eosinophils, B-lymphocytes, monocytes/macrophages and CD 34+ progenitor cells and has high affinity preferentially with LTD4 (Naik et al., 2005). The proinflammatory roles of cysLTs via CysLTR1 (Woszczek et al., 2005) have been certified by the observation that CysLT synthesis inhibitors or CysLTR1 antagonists not only reduced airway and/or blood eosinophilia but also downregulated bronchial constrictor responses to a variety of triggers. These improvements of baseline lung function in vivo and in vitro studies suggest that cysLTs contribute as potent factors to the development of asthma and some of the disease regulatory actions of cysLTs are mediated through CysLTR1 (Bjermer et al., 2002, Capra et al., 2006, Eum et al., 2003, Holgate et al., 2003, Nagata et al., 2003).

We hypothesized that LTD4, the potent mediator of asthma, might induce cytokines and/or chemokines that attract inflammatory cells directly or indirectly via CysLTR1. In this study, we investigated the effect of LTD4 via CysLTR1 on cytokine and chemokine expressions in human lung epithelial A549 cells, and also examined the inhibitory effect of CysLTR1 antagonist, MK-571 on the inflammatory role of LTD4.

Ⅱ. MATERIALS AND METHODS

A. Materials

Phosphate buffered saline (PBS) was made by laboratory protocol, MK-571, selective CysLTR1 antagonist, was purchased from Biomol (Plymouth Meeting, PA), RPMI 1640, Fetal Bovine Serum (FBS), and anti-biotics were purchased from GIBCO (NY, USA), Lipofectamine reagent was purchased from Invitrogen (Seoul, Korea), LTD4 was purchased from SIGMA (MA, USA), easy-BLUE total RNA extract reagent was purchased from iNtRON (Daejeon, Korea), MMLV reverse transcriptase and RNasin were purchased from Promega (Madison, WI, USA), QIAGEN plasmid maxi and midi prep kit were purchased from QIAGEN (QIAGEN, Hilden, Germany), Human Quantikine for CCL2/MCP-1 and CXCL10/IP-10 were purchased from R&D Systems (TECHNE Corporation, MN, USA), Human cytokine superarray assay was purchased from Superarray (Bioscience corporation, MD, USA), rabbit anti-human CysLTR1 polyclonal antibody, rabbit anti-human IP-10 polyclonal antibody and goat anti-rabbit IgG conjugated to FITC were purchased from Santa Cruz (CA, USA), ABI 7500 real-time RT-PCR machine and Power SYBR Green PCR master mix were purchased from Applied Biosystems (CA, USA).

B. Cell culture and transfection of CysLTR1 gene

A549 cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 100U/ml of penicillin G sodium, and 100ug/ml of streptomycin sulfate

(penicillin-streptomycin, GIBCO) and cultured at 37℃, and 5% CO_2 in humidified incubator. 2 X 10^5 Cells were seeded in each well of 6-well plate for 1 day prior to transfection so that they were attached with 60~70% confluency. To overexpress CysLTR1 in A549 cells, transfections were carried out in serum free medium using 5ul of Lipofectamine transfection reagent(Invitrogen), and 0.75ug of CysLTR1 construct and 0.75ug of 3xHA(Hemaglutanin) tagged CysLTR1 construct DNA per well according to the manufacturer's protocol. 5hrs after transfection, cells were added FBS and cultured for 24hrs to express transfected CysLTR1 genes.

C. Treatment of LTD4 and MK571

After transfections, cells were starved with serum free media and arrested for 18 hrs before being treated with 100nM of MK571, selective CysLTR1 antagonist, and/or 100nM of LTD4. In case of treatment of MK571, it was pretreated for 3hrs and removed by being changed into new plain RPMI 1640 medium (treatment of MK571 for 3hrs was more effective to block CysLTR1 than for 18 hrs) . To harvest all cells of each well at the same time, cells were treated with 100nM of LTD4 for 8, 6, 4, 2, 1 and 0.5 hrs before harvest respectively. Cells and supernatant of each well were kept for real-time PCR and/or western blotting, and ELISA respectively. For real-time PCR, cells were added 1ml of easy-BLUE (Intron) directly after removing supernatants. For western blotting, cells were harvested with 0.25% Trypsin-EDTA solution (Sigma) , washed in phosphate-buffered saline and lysed in lysis buffer or frozen at -78℃

D. RT-PCR analysis for CysLTR1 mRNA expression level

Total RNA was extracted from cells by using Easy-Blue reagent (Intron Biotechnology, Korea) according to the manufacturer's instructions. After denaturation of RNA and annealing with oligo dT 18mers (72℃, 5min), cDNA was synthesized from 2.5ug of RNA using 200U of MMLV-RTase (Promega) under optimized reaction conditions (RT buffer : 50mM pH 8.3Tris-HCl, 75mM KCl, 3mM MgCl2, 10mM DTT, 2mM dNTPs, 0.5U/ul RNase inhibitors (RNasin, Promega)) at 42℃ for 1hr and extended at 70℃ for 10min. To confirm the synthesis of cDNA or normalize the concentration of cDNA, β-actin RT-PCR was performed with primers 5'-TCCTTCTGCATCCTG TCGGC as forward primer and 5'-CAAGAGAT GGCCACGGCTGC as reverse primer using 0.03U of Taq polymerase (Solgent) under the following buffer and PCR condition (PCR-buffer : 20mM pH 8.4 Tris-HCl, 75mM KCl, 0.2mM dNTPs, 2mM MgCl2, 0.2uM forward and reverse primers, PCR condition : 22 cycles, denaturation : 95℃, 30sec ; annealing : 58℃, 30sec ; extension : 72℃, 30sec) using DNA Engine Thermal cycler (Bio-rad). Specific primers of target genes (CysLTR1, F : TGACCGCTGCCTTTTTAGTC, R : GAGAGGGTCAAAGCAACAA TTG ; MCP-1, F : TGCAGCTAACTTATTTTCCC, R : AGAACTGTGGTTCAAGAGGA ; CXCL10, F : CTAGAACCGTACGCTGTACCT, R : TCAGACATCTCT TCTCACCC) were designed in their own coding regions

E. Human inflammatory cytokines superarray assay

cDNA from A549 and CysLTR1-tranfected A549 cells was prepared for template. Assay was performed using ABI 7500 real-time RT-PCR following the protocol provided by the

manufacturer (SuperArray Bioscience Corporation, USA). The assay plate contains 84 kinds of human cytokines primers and 5 different housekeeping genes primers for control. The results were analyzed by $2^{-\Delta\Delta Ct}$ method using ABI 7500 prism software.

F. Quantitative Real-time RT PCR for CysLTR1, MCP-1 and IP-10

Quantitative real-time RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems). Amplification conditions were 95℃ for 15min as the first step, 45 cycles of denaturation at 95℃ for 15sec, annealing at 58℃ for 30sec, and extension at 72℃ for 33sec. For relative quantitation, we used a method that compares the amount of target genes normalized to an endogenous reference gene such as β-actin. The formula was 2-ΔΔCt, representing the n-fold differential expression of a specific gene in a treated sample compared with the control, where Ct is the mean of threshold cycle, ΔCt was the difference in the Ct values for the target gene and the reference gene, β-actin (in each sample), and ΔΔCt represents the difference between the Ct from the control and each datum. Before using this method, we performed a validation experiment comparing the standard curve of the reference and the target to show that efficiencies were equal. Because the annealing temperatures among reference gene and target genes, all real-time RT-PCR experiments in the same batch could be performed at the same time. Used primers were same with those for RT-PCR.

G. Measurement of released cytokines by ELISA

The releasing concentrations of MCP-1 and IP-10 in the supernatant fluid were

determined with ELISA specific kit (R&D Systems, Minneapolis, MN, USA). The detection limits for MCP-1 and IP-10 were 15.6pg/ml and 7.8pg/ml respectively. It was determined the optical density of each well within 30min, using a microplate reader set to 450nm.

H. Measurement of CysLT1 receptor expression on the membrane by flow cytometry

Cells were harvested using trypsin-EDTA , washed in PBS buffer and transferred to 5ml polystyrene round-bottom tube. For flow cytometry, cells were centrifuged at 1500rpm for 3min at 4℃ each time. And they were fixed with 4% paraformaldehyde-PBS for 30min at 4℃ and permeabilized with 0.3% Triton X-100-PBS supplemented with 5% FBS for 30min at 4℃. Cells were then labeled with a 1:100 dilution of an anti-CysLTR1 or anti-HA antibody in permeabilizing Triton X-100 solution for 40min at 4℃ and washed twice in PBS to remove unattached antibodies. And then cells were incubated with a 1:200 dilution of an anti-IgG conjugated to FITC in permeabilizing Triton X-100 solution for 40min at 4℃ and washed twice in PBS to remove unattached antibodies and get clear signal. For the last time, cells were resuspended in 200ul of PBS

Ⅲ. RESULTS

A. Cytokines / chemokines induced by LTD4 in A549 cells

As a first step in assessing the effect of LTD4 on CysLTR1 expression, we analyzed mRNA expression level of CysLTR1 by real-time RT-PCR in various cell lines including U937 (Human leukemic monocyte lymphoma cell), differentiated U937 (macrophage-like cell), THP-1 (human monocytes) and A549 cells(human lung epithelial cells). Among these cell lines, CysLTR1 was prominently increased by stimulation with 100nM of leukotriene D4(LTD4) with time dependency in A549 cells and peaked at 4hrs after stimulation (Fig. 1A). This result was reproduced in the triplicate experiments (Fig. 1B).

To search candidate inflammatory cytokines / chemokines which are sensitively responsible for LTD4, human inflammatory cytokines superarray assay containing 84 genes was performed. After stimulation of 100nM of LTD4 on A549, the cells were harvested in different time (0, 2, 4, and 6hrs) and then examined the expression levels of 84 inflammatory cytokines by real-time RT-PCR (Fig. 2.). CRP and CXCL10 were peaked at 2hr after LTD4 treatment (Fig. 2A.), CCL8, CCR8, CXCL6, and IL13 at 4hr (Fig. 2B.), and CCL2, CCL25, CCL4, IL8RB and LTA at 6hr (Fig. 2C.). Among them, MCP-1 and IP-10 were selected as target genes due to their biological roles in asthma. For MCP-1(CCL2) and IP-10(CXCL10) genes, gene-specific primers were designed respectively (Table 1.) and the mRNA expression levels were examined by real-time RT-PCR.

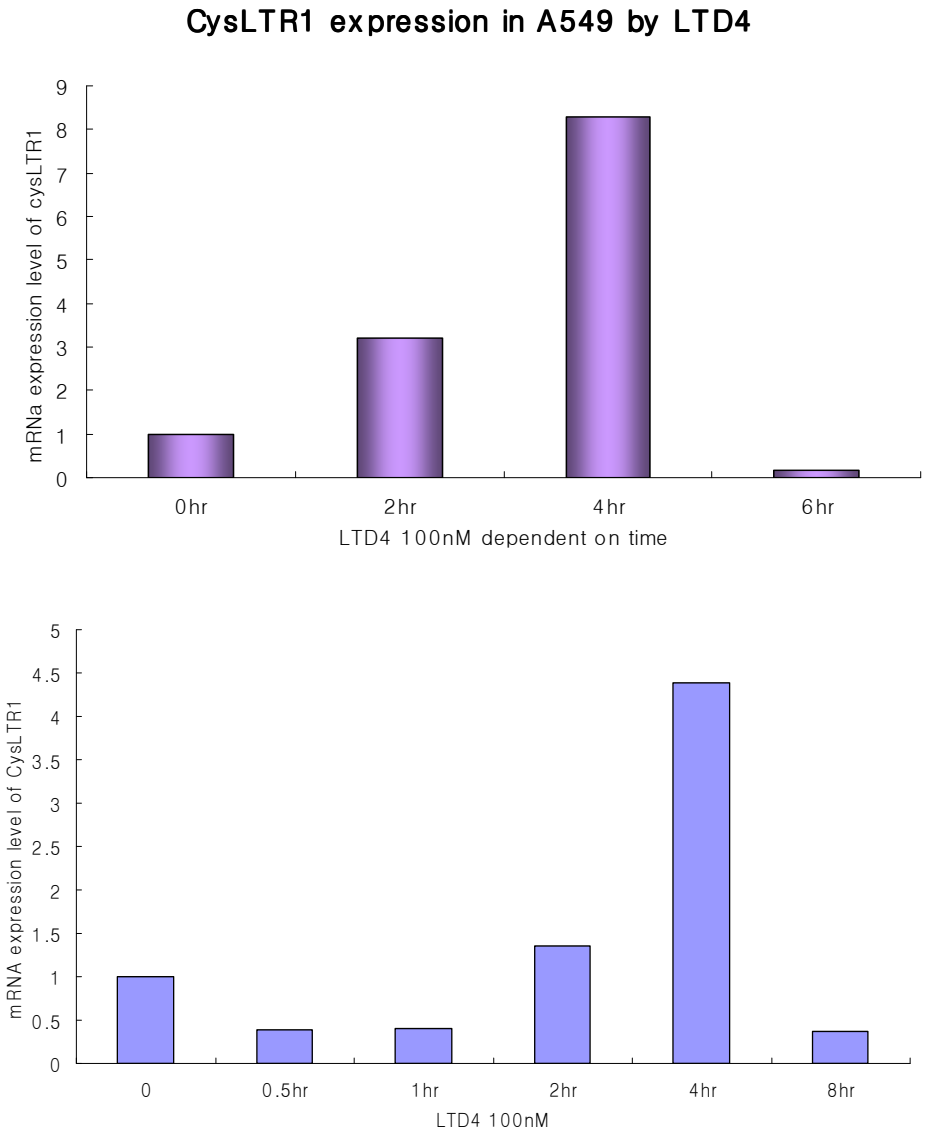

Fig. 1. CysLTR1 mRNA expression level using RT-PCR and real-time PCR.

(A) CysLTR1 mRNA expression level was dramatically increased in A549 cells after 4hrs of LTD4. $2X10^5$ cells / well in 6-well plate were cultured for 24 hours and arrested for 18 hours before treatment with LTD4 100nM for 0, 2, 4, and 8hours. (B) Same experiment as (A) was repeated to be confirmed and the result showed a similar tendency.

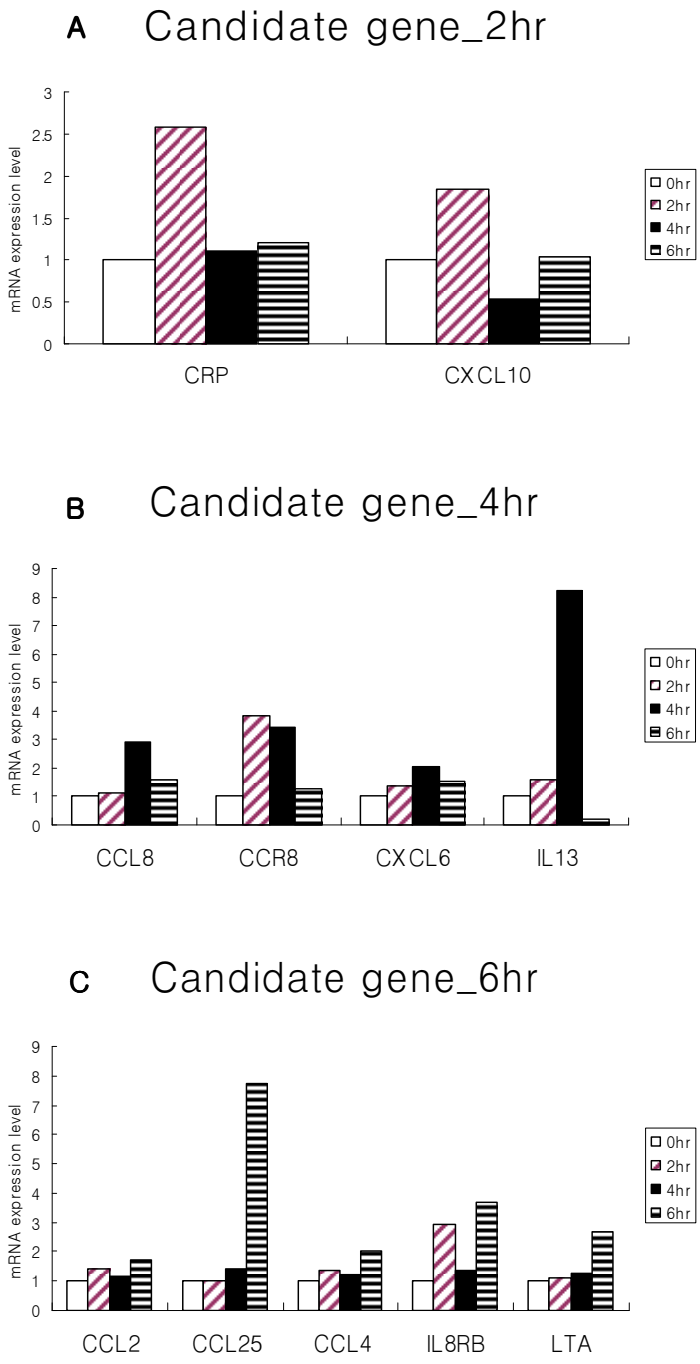

Fig. 2. The results of human inflammatory cytokines superarray using real-time PCR.

Differential expressions of several cytokines were found in A549 cells treated with LTD4

100nM dependent upon time. 2(A), 4(B) and 6hr(C)-treated cells were normalized to untreated cells. (CRP, C-reactive protein precursor ; CXCL10 , IP-10 (Interferon-ɣ-inducible Protein 10kDa) ; CCL8, Monocyte chemoattractant protein 2 ; CCR8, C-C chemokine receptor type 8 ; CXCL6, granulocyte chemotactic protein 2 ; IL13, Interleukin-13 precursor ; CCL2, MCP-1 (Monocyte Chemoattractant Protein – 1) ; CCL25, Thymus expressed chemokine, Ck beta-15 ; CCL4, Macrophage inflammatory protein 1-beta, Lymphocyte activation gene 1 protein ; IL8RB, High affinity interleukin-8 receptor B ; LTA, Lymphotoxin-alpha precursor, Tumor necrosis factor ligand superfamily member 1)

Table 1. List of primers of candidate genes

Gene /Aliases	Accession number Chromosome/cytoband	primer sequence	size
CCL2 MCP-1 (Monocyte Chemoattractant protein 1)	NM_002982 17q11.2-q12	F : TGCAGCTAACTTATTTTCCC R : AGAACTGTGGTTCAAGAGGA	209bp
CXCL10 10 kDa interferon- gamma-induced protein	NM_001565 4q21	F : CTA GAA CCG TAC GCT GTA CCT (21BP) R : TCA GAC ATC TCT TCT CAC CC (20BP)	153bp
CysLTR1 Cysteinyl leukotriene receptor 1	NM_006639 Xq13.2-q21.1	F : TGACCGCTGCCTTTTTAGTC R : GAGAGGGTCAAAGCAACAATTG	170bp
CCL16 SMALL INDUCIBLE CYTOKINE SUBFAMILY A, MEMBER 16	NM_004590 17q11.2	F1 : CAA GGA GAC TAG TGG TGG GAT A (22bp) R1 : TGA TGT ACT CTT GGA CCC AGT C (22bp)	126bp
CCL23 Macrophage inflammatory protein 3	NM_005064 17q12	F1 : GGA GAA AGA TTG GTC CTC AGA T (22bp) R1 : CGT TTC AAA GTA ACT CTC CAG G (22bp)	128bp
CXCL11 Interferon-inducible T-cell alpha chemoattractant	NM_005409 4q21.2	F1 : GTG CTA CAG TTG TTC AAG GC (20BP) R1 : CTT GGG TAC ATT ATG GAG GC (20BP)	118bp
CXCL12 Pre-B cell growth-stimulating factor	NM_000609 , NM_199168 10q11.1	F1 : CCT GAG CTA CAG ATG CCC ATG C (22bp) R1 : CAG GTA CTC CTG AAT CCA CT (20bp)	175bp
CXCL13 B lymphocyte chemoattractant	NM_006419 4q21	F1 : CCC TAG ACG CTT CAT TGA TCG A (22bp) R1 : TAT CCA TTC AGC TTG AGG GTC C (22bp)	132bp
CXCL14 Small inducible cytokine B14 precursor	NM_004887 5q31	F1 : CCC TAG ACG CTT CAT TGA TCG A (22bp) R1 : TAT CCA TTC AGC TTG AGG GTC C (22bp)	121bp
IL8RA interleukin 8 receptor, alpha	NM_000634 2q35	F1 : TTG GCC GAC CTA CTC TTT GC (20bp) R1 : CAA CAG CAG GAT GCC ACT GT (20bp)	142bp
IL1F6 interleukin 1 family, member 6 (epsilon)	NM_014440 2q12-q14.1	F1 : CTC ATA GCA GTC CCG AGG AA (20bp) R1 : CGG GTT GGT TGT ACA AAT CC (20bp)	209bp
ICEBERG Caspase-1 inhibitor Iceberg	NM_021571 11q21-q22	F1 : GAC ACT GTC ATG GAT AAG GC (20bp) R1 : TGA GGG TCT TCT TCA CAG AG (20bp)	104bp
CCR5 chemokine (C-C motif) receptor 5	NM_000579 3p21.31	F1 : AGG GCT GTG AGG CTT ATC TTC (21bp) R1 : TGG TCC AAC CTG TTA GAG CTA C (22bp)	143bp
IL13 Interleukin-13 precursor	NM_002188 5q31	F1: CTCATGGCGCTTTTGTTGAC (20bp) R1 : ACACGTTGATCAGGGATTCCA(21bp)	213bp

B. MCP-1 and IP-10 induced by LTD4 in A549 cells

To investigate the effect of LTD4 on MCP-1 and IP-10, additional experiments were performed. First of all, mRNA expression of MCP-1 after LTD4 stimulation was evaluated using real-time RT-PCR (Fig. 3.) MCP-1 mRNA level was 3 fold increased after 1hr treatment of LTD4 in A549 cells and then tailed out. Released MCP-1 level was gradually induced by LTD4 treatment (Fig. 4.) but the peaked point wasn't observed exactly so it needs to extend the observation time. Same purposed experiments were performed for IP-10 (Fig. 5.) mRNA expression of IP-10 was induced after 1hr of LTD4 treatment but not as much as MCP-1(Fig. 5.). Released protein level of IP-10 was also analyzed in the supernatant but the concentration of IP-10 is less than detectable limit (data not shown).

C. mRNA and protein expression of transfected CysLTR1

In a further series of experiments, CysLTR1 overexpressed A549 cells were prepared by transient transfection with pCMV-CysLTR1 construct and then treated with 100nM of LTD4. Overexpressed mRNA level of CysLTR1 gene after transfection was confirmed by RT-PCR (Fig. 6.). The surface expression of CysLTR1 was also confirmed by flow cytometry (Fig. 7.). These results indicate that transfected CysLTR1 can be highly expressed in both transcriptional and translational levels and then efficiently localized as a functional receptor in plasma membrane.

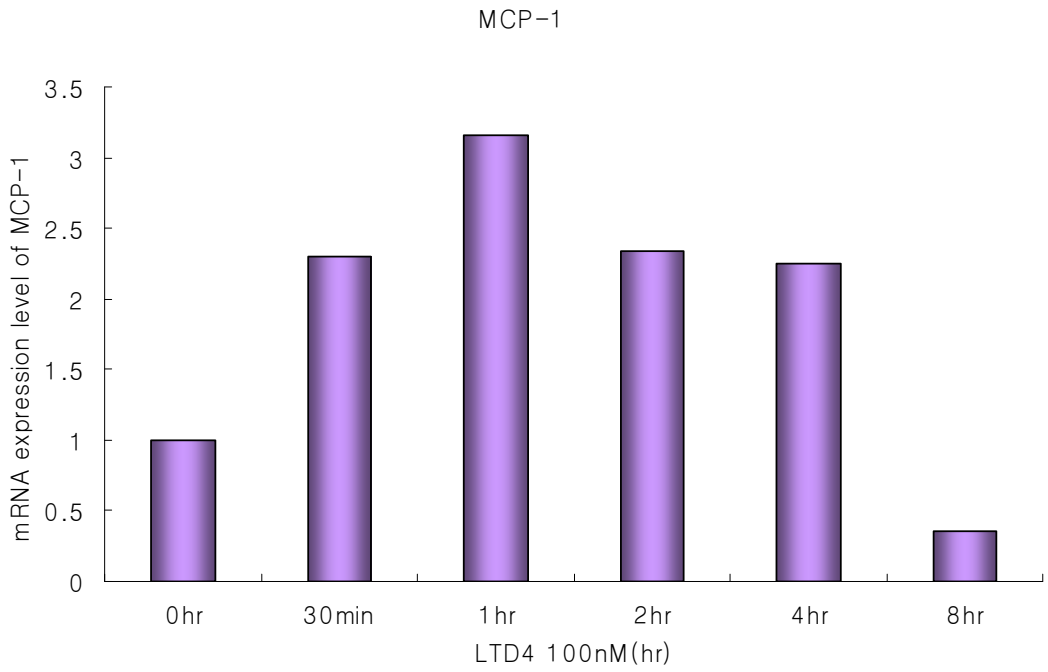

Fig. 3. Effect of LTD4 on MCP-1 mRNA level in A549 cells.

mRNA of MCP-1 was expressed differentially in A549 cells treated with LTD4 100nM dependent on time and measured by using real-time RT-PCR. (All cells were arrested for 18 hrs before LTD4 treatment)

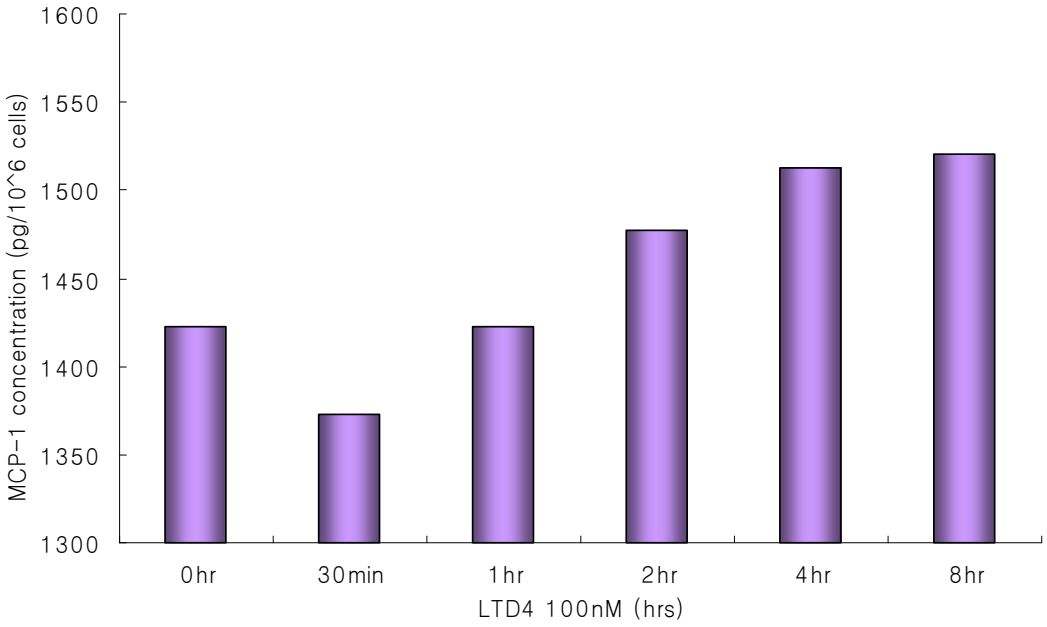

Fig. 4. Effect of LTD4 on MCP-1 released from A549 cells.

Released MCP-1 in cell supernatant was measured in the supernatant of LTD4-treated A549 cells by ELISA. (All cells were arrested for 18 hrs before LTD4 treatment)

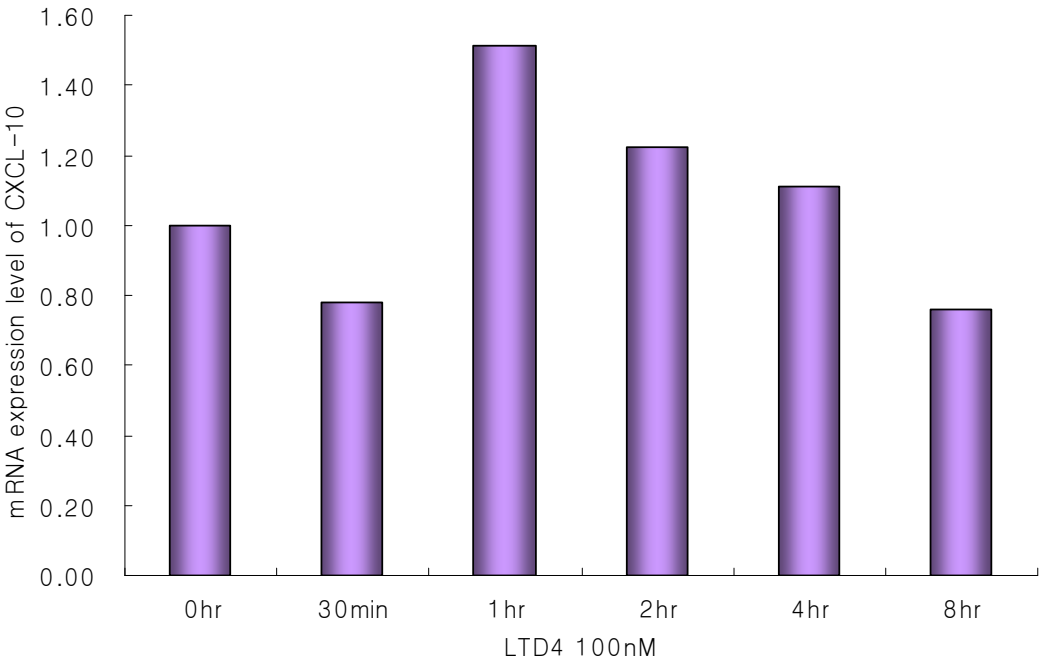

Fig. 5. Effect of LTD4 on the production of CXCL10 (IP-10) in A549 cells.

mRNA of CXCL10 (IP-10) was expressed differentially in A549 cells treated with LTD4 100nM dependent on time and measured by using real-time RT-PCR. (All cells were arrested for 18 hrs before LTD4 treatment)

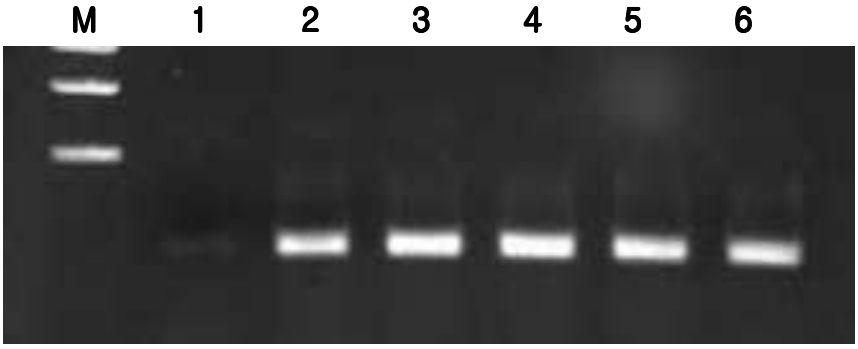

Fig. 6. mRNA expression of CysLTR1 after CysLTR1 transfection in A549 cells.

A549 cells were transfected with pCMV-CysLTR1 construct and then examined the mRNA level by RT-PCR. (M : 1Kb ladder , 1 : A : A549 , 2 : AC : CysLTR1 overexpressed A549 , 3 : ACL30min : CysLTR1 overexpressed A549 + LTD4 100nM 30min , 4 : ACL1hr : CysLTR1 overexpressed A549 + LTD4 100nM 1hr , 5 : ACL2hr : CysLTR1 overexpressed A549 + LTD4 100nM 2hr , 6 : ACL4hr : CysLTR1 overexpressed A549 + LTD4 100nM 4hr)

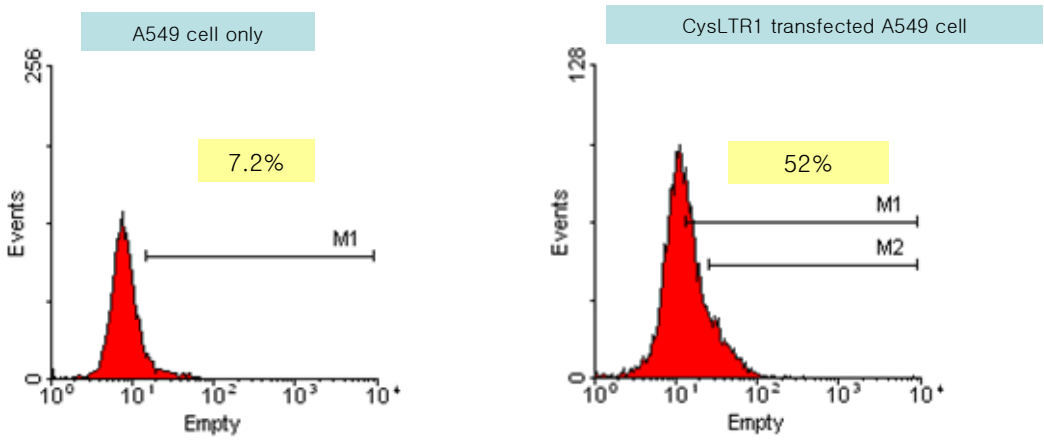

Fig. 7. Flow cytometric analysis of CysLTR1 surface expression.

After transfection, the localization of CysLTR1 on the cell membrane was confirmed by flow cytometry. Cells were labeled with anti-CysLTR1 Ab and stained with anti-IgG Ab conjugated to FITC.

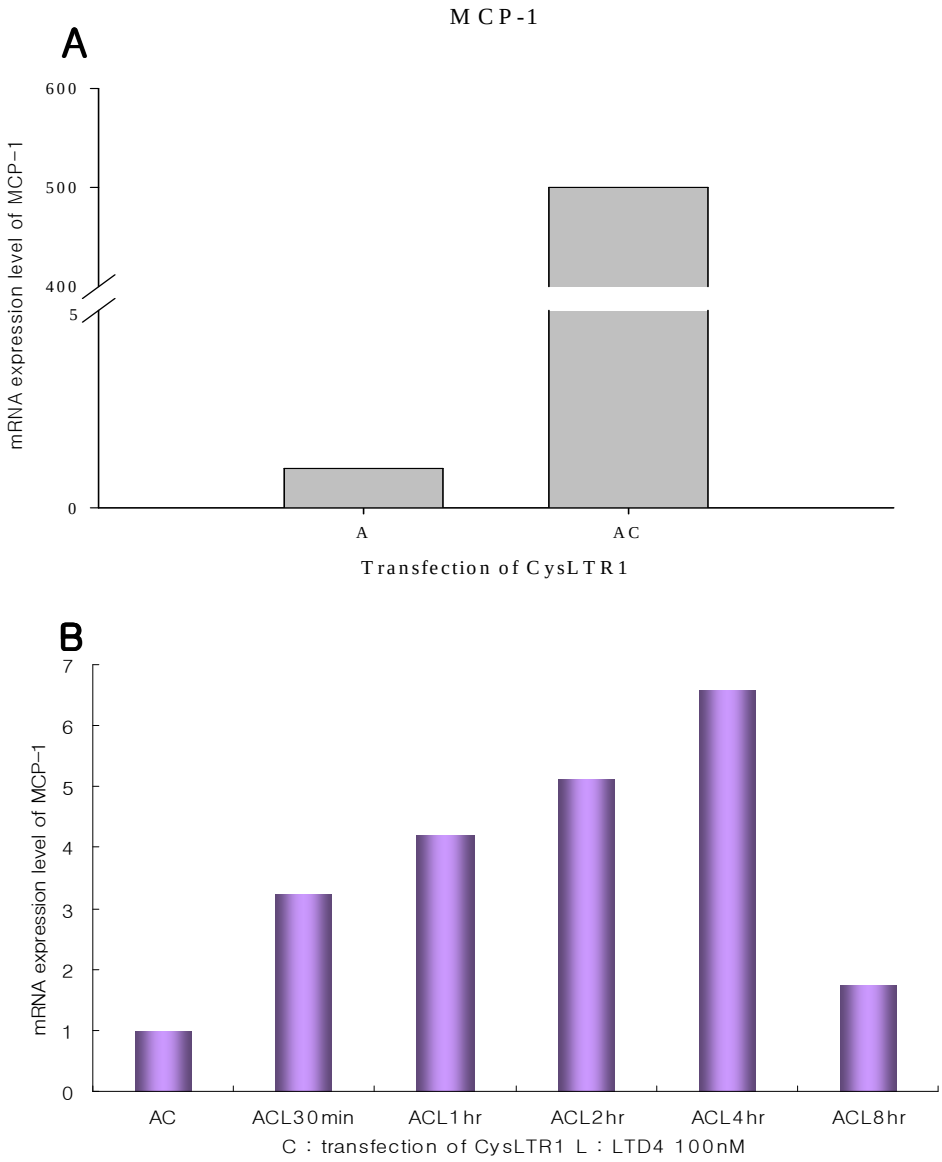

Fig. 8. Effect of LTD4 on CCL2 (MCP-1) in CysLTR1 -overexpressed A549 cells.

mRNA of MCP-1 was expressed differentially in A549 cells transfected with CysLTR1 and treated with LTD4 100nM dependent on time, and measured by real-time RT-PCR. After cultured for 24hrs, A549 cells were transfected with pCMV-CysLTR1 construct using lipofectamine for 5hours in serum free media and added FBS supplemented media for 24hrs.

And then cells were arrested for 15hrs before treatment of 100nM of LTD4. (A) After transfection of CysLTR1, mRNA expression of MCP-1 is significantly enhanced as much as 500-fold. (B) MCP-1 was upregulated gradually by LTD4 and maximally expressed at 4hrs and tailed out. (All cells were arrested for 18 hrs before LTD4 treatment)

D. Enhancement of MCP-1 and IP-10 by overexpressed CysLTR1

To exam whether LTD4 affects MCP-1 and/or IP-10 in CysLTR1-overexpressed A549 cells, mRNA and protein level of MCP-1 and IP-10 were analyzed. Interestingly, we found MCP-1 mRNA level was dramatically augmented as much as 400-fold over for transfected CysLTR1 alone (Fig. 8A). After LTD4 treatment on CysLTR1-overexpressed A549 cells, mRNA level of MCP-1 was gradually increased and peaked at 4hr, and tailed out at 8hr.

For released protein level of MCP-1, however, unexpected results were shown (Fig. 9.). While mRNA level of MCP-1 was augmented by overexpressed CysLTR1, released protein was rather decreased (data not shown). In addition, the effect of LTD4 on protein was also weak than that on mRNA. mRNA expression of IP-10 was also shown in similar tendency as that of MCP-1 in CysLTR1 overexpressed A549 (Fig. 10A.). It was enhanced by exaggerated CysLTR1, however, the mRNA level of IP-10 was slightly induced by LTD4 treatment. IP-10 protein was also augmented according to overexpressed CysLTR1. Even though the effect of LTD4 on IP-10 transcription level was weak (Fig. 10B.), LTD4 dramatically increased the released IP-10 protein level (Fig. 11.). When comparing to released MCP-1 protein, IP-10 is affected by overexpressed CysLTR1 even in translational level.

E. Inhibitory effect of MK-571 on MCP-1 and IP-10

Although MCP-1 and IP-10 seemed to be upregulated by LTD4 and CysLTR1 overexpre-

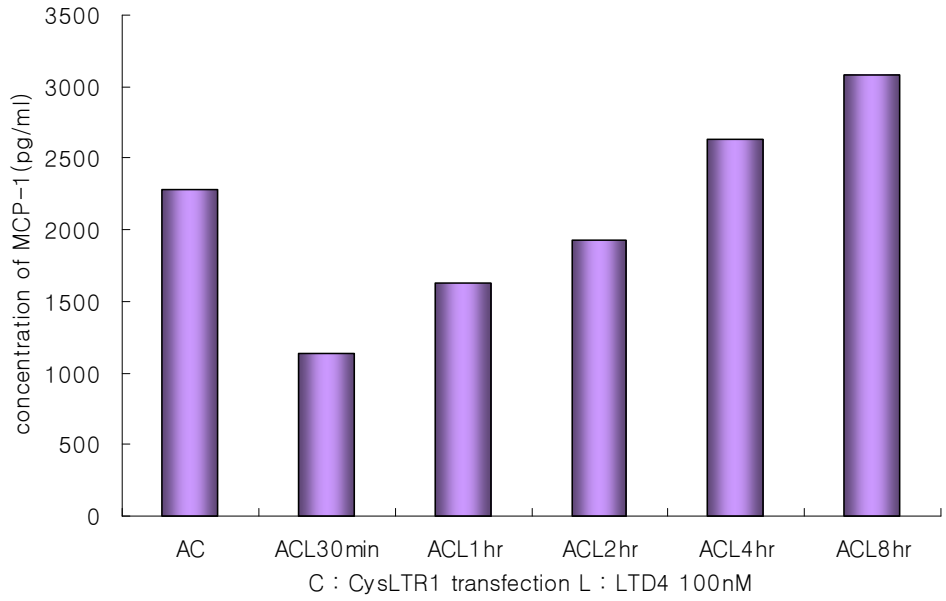

Fig. 9. Effect of LTD4 on releasing MCP-1 in CysLTR1-overexpressed A549 cells.

Released MCP-1 in cell supernatant was measured by ELISA. (All cells were arrested for 18 hrs before LTD4 treatment)

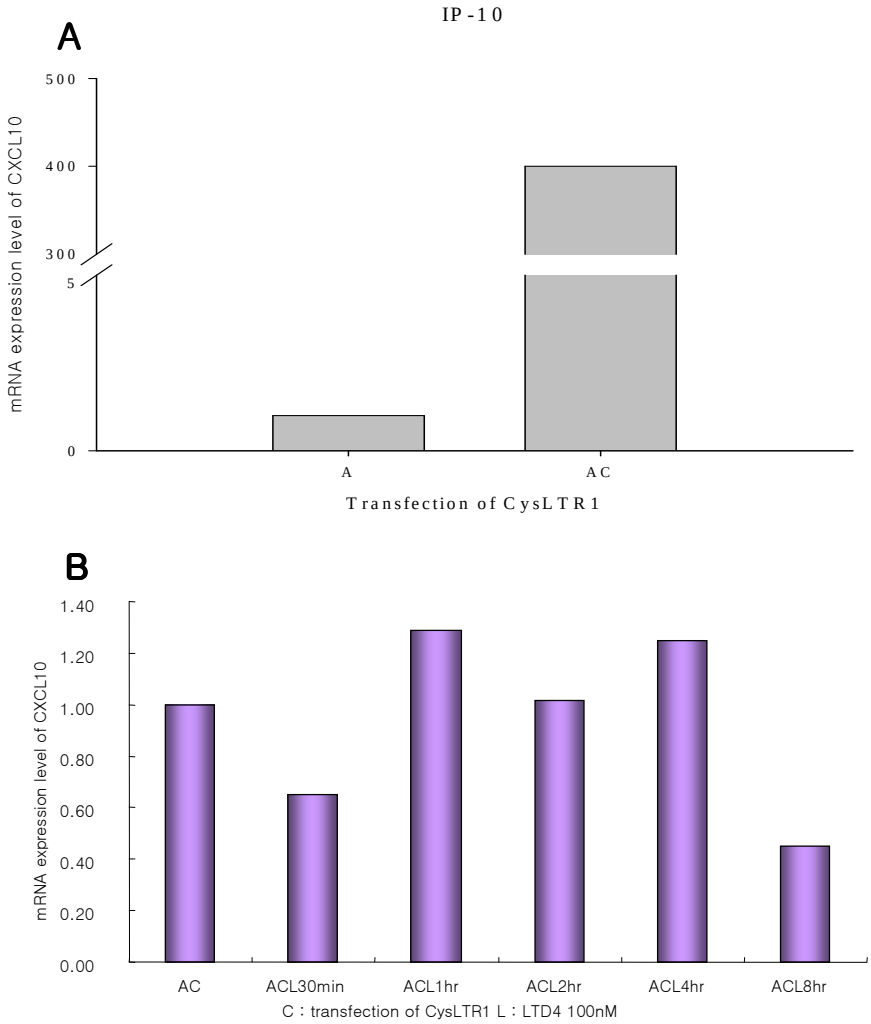

Fig. 10. Effect of LTD4 on the production of IP-10 in CysLTR1-overexpressed A549 cells. mRNA of MCP-1 was expressed differentially in A549 cells transfected with CysLTR1 and treated with LTD4 100nM dependent on time, and measured by using real-time RT-PCR. (All cells were arrested for 18 hrs before LTD4 treatment)

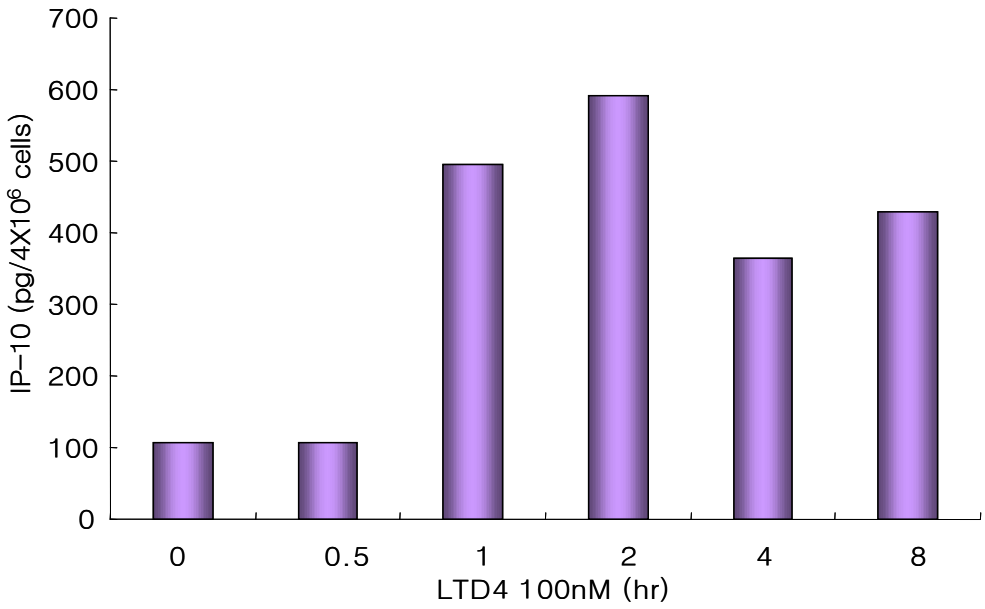

Fig. 11. Effect of LTD4 on releasing IP-10 in CysLTR1 overexpressed A549.

Released IP-10 in cell supernatant was measured by ELISA. (All cells were arrested for 18 hrs before LTD4 treatment)

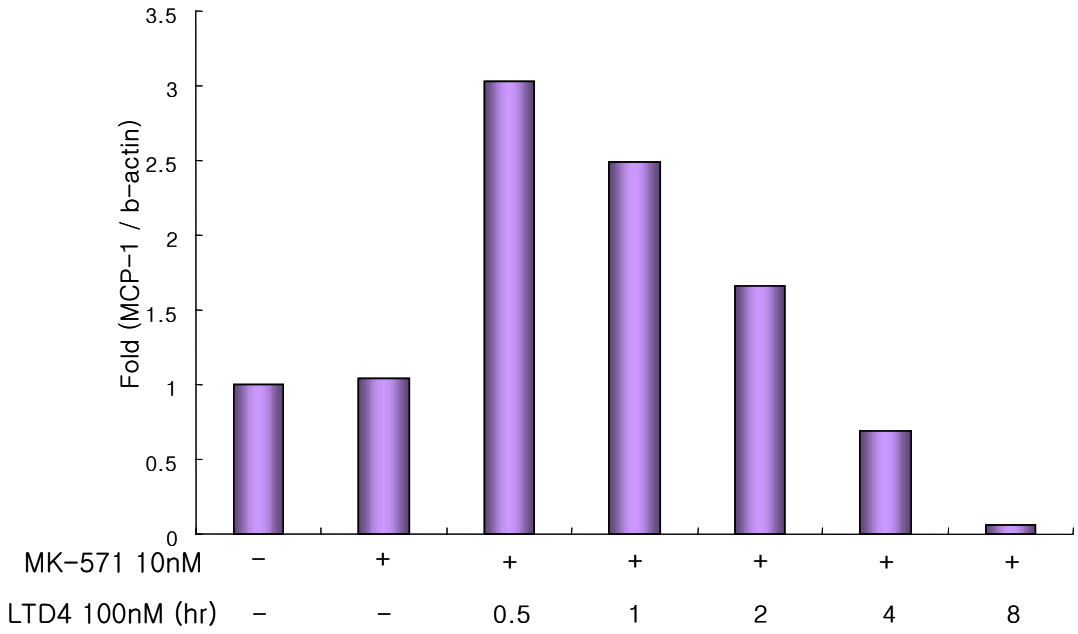

Fig. 12. Effect of MK571 on mRNA expression of MCP-1 in CysLTR1 overexpressed A549. Cells were pretreated with MK571, CysLTR1 antagonist, for 3hrs and stimulated with LTD4 100nM dependent on time. (All cells were arrested for 18 hrs before LTD4 treatment)

-ssion, it wasn’t certain that induction of MCP-1 and IP-10 by LTD4 are mediated through CysLTR1. To confirm whether LTD4 acts on the production of MCP-1 and IP-10 via CysLTR1, selective CysLTR1 antagonist, MK571 was treated in CysLTR1-overexpressed A549 cells and then mRNA expression levels of MCP-1 and IP-10 were analyzed. In case of MCP-1, as illustrated in Fig. 12. , MK571 didn’t inhibit the effect of LTD4 at all. In contrast, IP-10 expression was completely suppressed by pretreatment of the cells with MK571 despite being recovered by LTD4 100nM in 30min (Fig. 13.). Involvement of the CysLTR1 was confirmed by significant abrogation of IP-10 production in the presence of MK571 while nothing happened for MCP-1 (Fig.12., 14.). These observations suggest that the augmentation of IP-10 expression is mediated via CysLTR1 whereas MCP-1 isn’t.

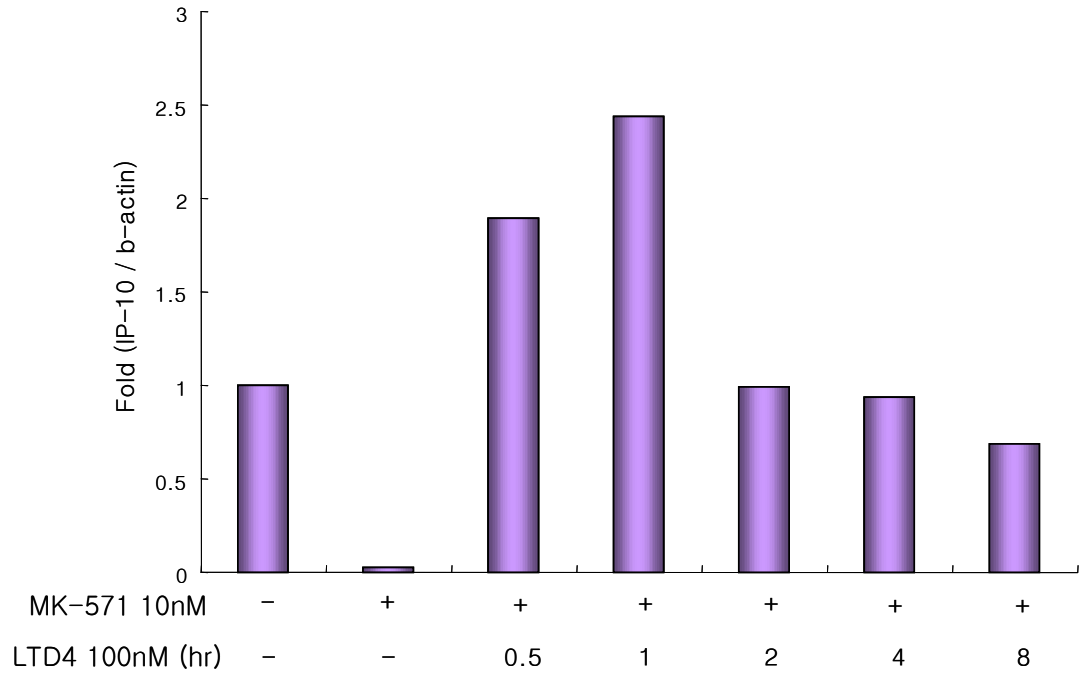

Fig. 13. Effect of MK571 on mRNA expression of IP-10 in CysLTR1 overexpressed A549. Cells were pretreated with MK571, CysLTR1 antagonist, for 3hrs and stimulated with LTD4 100nM dependent on time. (All cells were arrested for 18 hrs before LTD4 treatment)

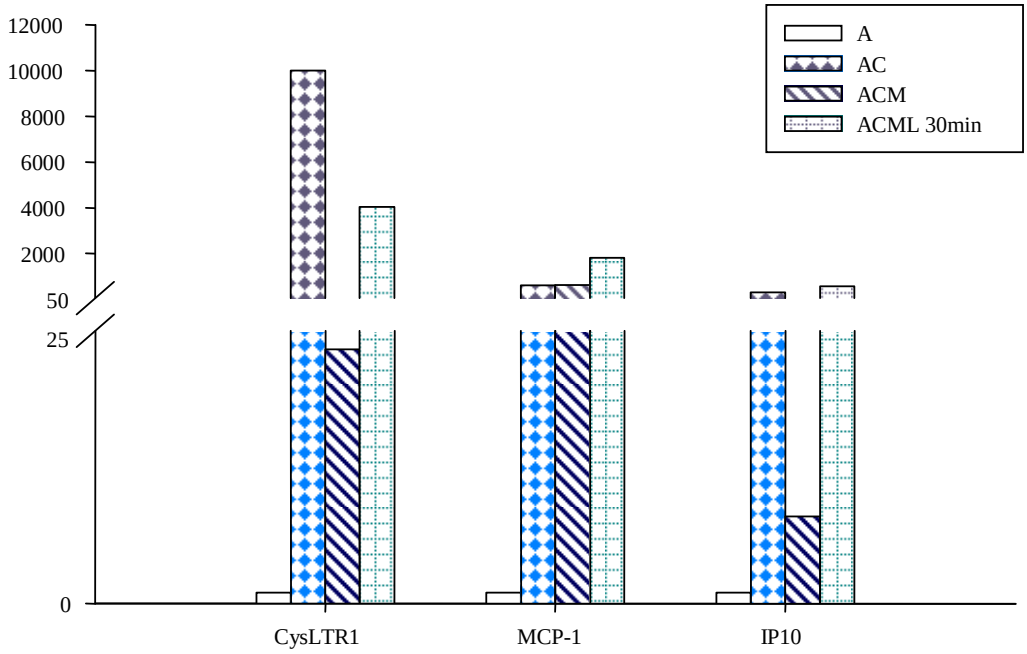

Fig. 14. Dependency on CysLTR1.

A549 Cells were transfected with CysLTR1 construct, pre-treated with MK571 CysLTR1 antagonist, for 3hrs, and stimulated with LTD4 100nM for 30min.

Ⅳ. DISCUSSION

The cysLTs, particularly LTD4, is a potent lipid mediator that has been implicated in the pathogenesis of inflammatory processes, preferentially asthma characterized as bronchoconstriction, mucus secretion, and airway hyperresponsiveness. It has been shown that IL-4, the prototypical Th2 type cytokine up- regulates direct chemotactic response to cysLTs by increasing expression of CysLTR1 on human monocytes/macrophages (Thivierge et al., 2001). We hypothesized that the proinflammatory mediator, LTD4 might induce cytokines and/or chemokines which are attractive to inflammatory cells directly or indirectly. Thus, this present work was initiated to study the regulatory mechanisms of cytokines / chemokines, finally leading to persist chronic inflammation in asthmatic patients by LTD4 via CysLTR1. In the present study, we found two chemokines, MCP-1 and IP-10 are prominently up-regulated by LTD4 in human lung epithelial A549 cells.

A549 cells are a kind of alveolar epithelial cells type II that produce surfactant and act as progenitors to replace injured alveolar epithelial cells type I. Thus, they are located at the boundary between the alveolar airspace and the interstitium, and ideally situated to regulate the recruitment and activation of different types of leukocytes through the production of cytokines / chemokines in response to inflammatory stimulation from the alveolar space (Pechkovsky et al., 2005). Recently, it has been suggested that alveolar epithelial cells secrete a variety of mediators, including proinflammtory cytokines and chemokines important for the recruitment of monocytes / macrophages and T cells into the lung

interstitium and alveolar space (Barrett et al., 1998, Kay, 1983, Koyama et al., 1997). In this study, A549 cells rarely expressed CysLTR1 mRNA and they were transfected with CysLTR1 CDS and/or stimulated with LTD4 to represent a condition that cysLTs and CysLTR1 are accumulated in the lungs of patients with asthma. Moreover, CysLTR1 was enhanced by its agonist LTD4 dependent on autocrine signaling, which means A549 cells are stimulated with LTD4 through CysLTR1 and release LTD4 which binds its own receptor CysLTR1, causing self-stimulation. Thus, overexpressed CysLTR1 might elicit more effective signaling to downstream cytokines / chemokines.

Chemokines play an important role in the pathophysiology of asthma and allergy (Hung et al., 2006). A study has reported that airway hyperreactivity could be mediated by allergen-induced, as well as directly instilled-MCP-1 through CCR2, and significantly attenuated in CCR2-/- mice (Campbell et al., 1999). Besides, MCP-1 plays a key role in monocyte recruitment by integrin activation and by promoting migration to the vessel wall (Ashida et al., 2001) and it has been shown that its increased production is induced by cysLTs (LTD4, LTC4) in IL-4 primed THP-1 cells (Woszczek et al., 2005). So, MCP-1 and its receptor CCR2 are potentially important therapeutic targets for the treatment of hyperreactive airway disease and for repair of injured bronchial epithelial cells in asthma (Campbell et al., 1999). Nuclear factor (NF) – kappa (κ) B plays a major role in regulation of inflammatory genes including cytokines, chemokines, inflammatory enzymes, adhesion molecules, and others. Therefore NF-κB is considered as a target for novel anti-inflammtory therapies in diseases such as asthma and COPD (Newton et al., 2002) . It has been reported that NF-κB DNA binding activity was induced by LTD4 through activation of CysLTR1 (Thompson et al.,

2006) and the promoter region of MCP-1 gene contains binding site of NF-κB (Hong et al., 2007). A previous study showed that LTC4, -D4, and –E4 induced MCP-1 in THP-1 human monocytes and CD14+ monocytes/macrophage (Ichiyama et al., 2005). In that study, the inhibitory effect of CysLTR1 antagonist pranlukast on MCP-1 were examined and resulted in almost complete blockade of production of MCP-1 by cysLTs in THP-1 cells and partial inhibition in CD14+ cells. In present study, we examined the effect of LTD4 on MCP-1 in A549 cells and observed that mRNA of MCP-1 was induced by LTD4 in A549 cells as in the previous report in THP-1 cells, and excessive CysLTR1 augmented MCP-1. We also examined the inhibitory effect of CysLTR1 antagonist MK-571 on MCP-1 in CysLTR1-overexpressed A549 cells. In this study, however, CysLTR1 antagonist didn't act as a inhibitor and didn't make any difference of mRNA expression level between in presence and in absence of MK-571 suggesting MCP-1 wasn't regulated via CysLTR1. And it was estimated that MCP-1 production is differently regulated according to cell type and is regulated by LTD4 partially, but might be mediated via CysLTR2, not CysLTR1.

IFN-induced protein of 10kDa (IP-10/CXCL10) is a member of CXCL class and target preferentially activated Th1 lymphocytes and natural killer cells through its receptor CXCR3 (Cole et al., 1998, Farber, 1997). IP-10 has been described in several cell types, including monocytes, keratocytes and neutrophils involved in the more severe form of asthma. Concentration of IP-10/CXCL10 was reported to be elevated in bronchoalveolar lavage fluid of atopic asthmatics following segmental allergen challenge (Bochner et al., 2003, Ying et al., 2005) and elevated numbers of cells expressing IP-10/CXCL10 mRNA and protein in bronchoalveolar lavage fluid and the bronchial mucosa have previously been detected in

asthmatics(Miotto et al., 2001, Ying et al., 2005). Similarly, It has been shown that in a murine model, IP-10 is up-regulated in allergic pulmonary inflammation and overexpressed IP-10 resulted in elevated eosinophil infiltration and IL-4 expression, and the airway hyperreactivity whereas IP-10/CXCL10 deficiency resulted in opposite effects (Medoff et al., 2002). And a study has reported MCP-1 and IP-10 are differently expressed and regulated in human alveolar epithelial cells type II ; MCP-1 is spontaneously expressed in alveolar epithelial cells type II whereas IP-10 is expressed in presence of IFN-γ (Pechkovsky et al., 2005). In present study, IP-10 was differentially expressed in response to LTD4 and elicited high expression by overexpressed CysLTR1 because LTD4 can affect IP-10 more strongly through excessive CysLTR1. The effect of LTD4 on IP-10 through CysLTR1 was evidenced by the inhibitory effect of MK-571 that blocked production of IP-10 in CysLTR1-overexpressed A549 cells, and production of IP-10 was recovered completely by LTD4 100nM in 30 minutes. They suggest IP-10 is regulated by LTD4 directly via CysLTR1. As noted above, NF-κB plays a key role as a regulator of inflammatory genes and the promoter region of IP-10 contains NF-κB and other transcription factor binding sites that are induced by LTD4 (Spurrell et al., 2005).

We demonstrated that IP-10 and MCP-1 are up-regulated by cysteinyl leukotriene D4 (LTD4) although it made a difference of effect respectively. Of interest, the expressions of IP-10 and MCP-1 were increased for overexpressed CysLTR1 alone. MK571, CysLTR1 antagonist inhibits the expression of IP-10 but not MCP-1 suggesting that IP-10 may be involved in CysLTR1 downstream directly, whereas other pathway may be involved in case of MCP-1.

Ⅴ. CONCLUSION

This study suggests that pro-inflammatory chemokine MCP-1(CCL2) and IP-10 (CXCL-10) are up-regulated by cysteinyl leukotriene D4 (LTD4), and mRNA expression levels of IP-10 and MCP-1 are increased for overexpressed CysLTR1 alone. In case of IP-10, released protein production as well as mRNA is augmented by LTD4 and CysLTR1. MK571, CysLTR1 antagonist inhibits the expression of IP-10 but not MCP-1, suggesting that IP-10 may be involved in downstream pathway of CysLTR1 directly whereas other pathway has to be suggested for MCP.

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-국문요약-

폐상피세포 A549에서 류코트리엔 제 1 수용체를 통한 케모카인의 발현에 미치는 류코트리엔 D4 의 영향

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연구배경 및 목적 : 류코트리엔 C4, D4 그리고 E4 (Leukotriene C4, D4, E4)와 같은 시스테인 류코트리엔 (cysLTs)은 지질 전염증 매개인자 (proinflammatory lipid mediator)로서, 세포막의 아라키돈산 (arachidonic acid)으로부터 5-리폭시제나아제 (5-lipoxygenase) 경로를 통해 생성된다. 이들은 기관지수축, 점액질 분비, 기도과민성, 혈관 과투과성 등을 야기하는 중요한 물질로 알려져 있고, 생체내에서 염증 반응을 개시하는 백혈구를 직,간접적으로 유인하는 역할을 하는데, 이는 G-단백결합수용체인 류코트리엔 제 1 수용체 (CysLTR1)를 통해 신호를 전달하는 것으로 알려져 있다.

폐의 상피세포 (Human lung epithelial cells) 인 A549에 IL-4나 IL-13을 자극함에 따라 CCL24, CCL26과 같은 케모카인 (chemokine) 의 생성을 조절할 수 있다는 보고는 있었으나, 류코트리엔과의 관계에 대한 보고는 아직 없었다.

이 실험의 목적은 폐 상피 세포인 A549에서 LTD4의 자극에 의해 조절되

는케모카인을 찾아보고, 그 물질들이 유도되는 것이 LTD4의 수용체인 CysLTR1을 매개로 하는지를 연구하여, 천식과의 관련성을 규명하는 것이다.

연구방법 : 류코트리엔 D4의 자극에 의해 시간에 따라 CysLTR1의 발현량에 차이를 보이는 세포주를 선정하기 위해 real-time RT-PCR 를 실시하였고, 그 결과 폐 상피 세포인 A549가 선정되었다. 0, 2, 4, 6 시간동안 LTD4에 의해 자극받은 A549세포에서 추출한 RNA 로부터 cDNA 를 얻어, 84가지의 염증관련 사이토카인/케모카인의 프라이머(primer)가 장착되어 있는 사이토카인 array를 가지고 real-time RT-PCR를 실시함으로써, LTD4에 의해 특정 시간에 유도되는 사이토카인을 선별하였다. 이 과정을 통해 선정된 CCL2 (MCP-1) 와 CXCL10 (IP-10) 의 유전자에 특이적으로 결합하는 프라이머를 제작, real-time PCR에 이용하였다. 먼저, A549에서 두 케모카인에 미치는 LTD4의 영향을 보기 위해 LTD4를 시간별로 처리한 A549 세포를 준비하였다. 그리고 CysLTR1이 과발현된 상태를 만들기 위해 pCMV vector 에 CysLTR1의 아미노산을 암호화 하는 부위(Coding region)을 재조합한 pCMV-CysLTR1 CDS 플라스미드를 A549 에 형질전환(transfection) 한다. 이 후 마찬가지로 시간별로 LTD4를 처리하여 CysLTR1이 과발현된 상태에서 MCP-1과 IP-10에 미치는 LTD4 의 영향을 보고자 했다. LTD4에 의해 유도되는 MCP-1과 IP-10이 실제 LTD4의 수용체인 CysLTR1을 매개로 하는 것인지를 알아보기 위해 CysLTR1의 길항제(antagonist)인 MK-571을 처리하고, LTD4를 처리해 보았다. 이렇게 각각 다른 상황에서 MCP-1과 IP-10의 mRNA와 분비되는 단백질이 어떻게 발현되는지 관찰하기 위해, mRNA의 경우 real-time RT-PCR을, 단백질의 경우 ELISA

를 실시하였다. 또한, 형질전환된 CysLTR1유전자가 mRNA 로 전사되는지 RT-PCR로 알아보았고, 단백질로 만들어지고, 수용체로서의 역할을 할 수 있도록 세포막으로까지 이동되는지 알아보기 위해 유세포 측정법 (flow cytometry)을 이용하였다.

결 과 : A549에 LTD4 100nM 을 처리하고 30분 만에 MCP-1 mRNA 의 발현이 증가되었고, IP-10의 경우 MCP-1 과 비교할 때 증가된 정도의 차이는 미비하나, 역시 LTD4 처리 1시간 후에 mRNA의 발현이 증가됨이 관찰되었다. A549에 CysLTR1을 형질전환 후 MCP-1과 IP-10의 mRNA 발현량을 측정한 결과, 두 케모카인 모두 각각 500 배, 300 배 정도씩 눈에 띄게 증가하였다. CysLTR1을 과발현시키고 LTD4 100nM를 처리했을 때와 단순히 LTD4만 자극했을 때의 A549에서 두 케모카인의 발현양을 비교했을 때, 각각 비슷한 경향으로 발현되는 것을 볼 수 있었다. 그러나 세포 밖으로 분비되는 단백질을 ELISA 방법으로 측정한 결과, MCP-1의 단백질의 농도는 CysLTR1을 과발현시킨 이후에 오히려 감소된 것을 볼 수 있었고, CysLTR1이 과발현된 A549에 LTD4를 처리한 이후에 차츰 그 양이 증가되었다. IP-10의 경우에는 mRNA 의 발현양상과 비슷하게, CysLTR1이 과발현 된 상태에서 단백질의 분비량도 100 배 가량 증가되었고, mRNA의 발현에 있어서 LTD4의 영향이 미비했던 것과 달리 LTD4 처리 후 단백질 분비량은 확연히 증가되는 것이 관찰되었다. 이러한 반응들이 실제 CysLTR1을 매개로 하는 것인지 알아보기 위해 MK-571을 처리해 보았다. CysLTR1을 과발현 시킨 A549 세포에 MK-571을 처리하고 MCP-1 과 IP-10의 mRNA 발현양을 측정한 결과, MCP-1은 MK-571 전과

후에 아무런 변화가 없었고, IP-10의 경우 MK-571을 처리하자 그 양이 현저히 감소되었다. 이후 LTD4를 처리했을 때 MCP-1은 종전의 발현 양상과 다름없이 발현되었지만, MK-571에 의해 현저히 감소되어 아무것도 처리하지 않은 A549 에서의 발현량 정도였던 IP-10의 경우, LTD4에 의해 완전히 회복되고, 또한 더욱 증가되는 것을 볼 수 있었다. 이로써, IP-10은 직접적으로 CysLTR1을 매개로 신호가 전달되고, 발현이 조절됨을 알 수 있고, MCP-1은 CysLTR1의 영향을 받긴 하지만 간접적으로 신호가 전달되는 케모카인인 것이 밝혀졌다. 형질전환된 CysLTR1유전자가 실제 세포막으로 이동하여 수용체로서의 역할을 담당할 수 있는지를 알아보기 위해 유세포 측정법으로 세포 표면의 수용체의 개수한 결과, 일반 A549의 세포막에 존재하는 수용체보다 약 7배 가량 많은 수용체가 CysLTR1 유전자를 형질전환 시킨 A549의 세포막에서 측정되었다. 이로써 과발현시킨 CysLTR1 유전자가 mRNA로 전사되는 수준에서 끝나는 것이 아니라 단백질로도 표현되고, 실제로 사용될 수 있음이 밝혀졌다.

결 론 : CCL2 (MCP-1)과 CXCL10 (IP-10)은 류코트리엔 D4 (LTD4)에 의해 mRNA 발현량이 증가되고, 류코트리엔 제 1 수용체 (CysLTR1)가 과발현된 조건 하에서 아무런 자극 없이도 발현량이 급격히 증가된다. 그러나 IP-10은 직접적으로, 반면 MCP-1은 간접적으로 CysLTR1의 영향을 받아 발현이 조절된다.

핵심어 : 천식, 류코트리엔 D4, 류코트리엔 제 1 수용체, 사이토카인, 케모카인, MCP-1, IP-10, 류코트리엔 제 1 수용체 길항제, MK-571