

Effect of Clarithromycin on the Expression of UL16-Binding Protein 2 in Human Cells

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ABSTRACT

Background Clarithromycin is a macrolide antibiotic that possesses anti-inflammatory and immunomodulatory properties. Although recent data suggests that macrolide antibiotics enhance *Pseudomonas aeruginosa* clearance from the lung, involving natural killer (NK) T cells in this process by activating the NKG2D-NKG2D ligand system, the precise underlying mechanism is still unclear. In this study, we examined the effect of clarithromycin on a potent NKG2D ligand, UL16-binding protein 2 (ULBP2), in the lung and its shedding mechanism.

Methods The gene expressions of ULBP2 and the shredder proteinases of ULBP2, a disintegrin and metalloproteinase domain 10 (ADAM10) and ADAM17, were measured using real-time PCR. The cell surface ULBP2 expression was measured by flow cytometry. The amount of solubilized ULBP2 (sULBP2) was measured using an ELISA. The activity of ADAM17 was examined by measurement of fluorescence intensity from the fluorescence resonance energy transfer peptide substrate cleaved by ADAM17.

Results Clarithromycin significantly induced transcription of ULBP2 and ADAM17 in both A549 and LCSC #2 cells, which endogenously express minimal and abundant levels of ULBP2, respectively. However, there was no significant change on transcription of ADAM10. The same tendency was observed when LCSC #2 cells were treated with tumor necrosis factor- α processing inhibitor-2 to inhibit ADAM17 activity. The amount of sULBP2 was significantly decreased in

both A549 and LCSC #2 cells by treatment with clarithromycin. Finally, clarithromycin significantly inhibited the activity of ADAM17 in LCSC #2 cells.

Conclusion These findings suggest that clarithromycin induces ULBP2 expression and reduces the amount of sULBP2, by possibly inhibiting the activity of the potent ULBP2-shedding enzyme ADAM17. Because these changes in ULBP2 and sULBP2 levels could activate NKT cells, this finding might indicate a novel mechanism by which clarithromycin improves the clearance of *P. aeruginosa* in chronic respiratory diseases.

Key words a disintegrin and metalloproteinase domain 17 protein; clarithromycin; *Pseudomonas aeruginosa*; UL16 binding protein 2

Clarithromycin is a member of the macrolide antibiotic class, potent and well-established antimicrobials with 14- and 15-member lactone rings that also possess anti-inflammatory and immunomodulatory properties.¹⁻³ The effect of clarithromycin is mainly attributed to inhibition of inflammatory cytokine and chemokine production. The most proverbial demonstrations of the anti-inflammatory and immunomodulatory effects of macrolide antibiotics were reported in the successful treatment of diffuse panbronchiolitis⁴ and cystic fibrosis.⁵ These successes expanded the evaluation of macrolide antibiotics for the treatment of other chronic inflammatory diseases in the lung.⁶

Chronic infection of the lung by pathogens such as *Pseudomonas aeruginosa* is a life-threatening problem for patients with diffuse panbronchiolitis,⁷ cystic fibrosis⁸ and other chronic inflammatory lung diseases. Recent data suggests that macrolide antibiotics enhanced the clearance of *P. aeruginosa* from lung, although they intrinsically do not have antimicrobial activity for this bacterium.⁹ This result is thought to be another beneficial effect of macrolide antibiotics for the treatment of lung diseases; however, the precise mechanism of clarithromycin-induced clearance of *P. aeruginosa* in these chronic diseases remains unclear.

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Abbreviations: ADAM, a disintegrin and metalloproteinase domain; FRET, fluorescence resonance energy transfer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor-like growth factor; IL, interleukin; MFI, mean fluorescence intensity; NK, natural killer; sULBP2, solubilized ULBP2; TAPI-2, TNF- α processing inhibitor-2; TGF, transforming growth factor; TNF, tumor necrosis factor; ULBP2, UL16-binding protein 2

Recent data also suggests that natural killer (NK) T cells play a central role in clearing *P. aeruginosa* from the lungs.¹⁰ NKT cells are a specialized type of T cell that share properties of both T cells and NK cells and are emerging as critical regulators of the immune response to infectious agents.^{11, 12} Additionally, NKT cells are thought to play a role in managing human infections such as cystic fibrosis.^{13, 14} Previous studies showed that the activities of NKT cells, CD8+T cells and NK cells are tightly controlled by the activation receptor NKG2D that is expressed on the cell surface of these immune effector cells.¹⁵ The ligands for NKG2D are generally not expressed in normal cells, but their expression is induced in infected¹⁶ or transformed¹⁷ cells that should be eliminated by the host immune system. NKG2D on the surface of immune effector cells recognizes its ligands expressed on the surfaces of target cells and subsequently augments the cytolytic activity of the immune effector cells to promote destruction and clearance of pathogen-infected cells. In line with this, recent data suggests that expression of NKG2D contributes to the pulmonary clearance of *P. aeruginosa*.^{16, 18} Therefore, this NKG2D-NKG2D ligand system is thought to play an essential role in host immunity during chronic lung infections.

UL16-binding protein 2 (ULBP2) is one of the ligands for NKG2D that is expressed on infected human lung epithelial cells.¹⁹ Recently, we demonstrated that cell surface ULBP2 is released in soluble form from the surface and the resultant soluble ULBP2 (sULBP2) reduces the cytotoxic activity of human peripheral blood mononuclear cells.¹⁹ Therefore, ULBP2 on the cell surface and sULBP2 may have crucial but opposite effects on the infection immunity of NKT, CD8+T and NK cells in the lung. However, the effect of clarithromycin on these molecules has not been explored. In the present study, we evaluated the effect of clarithromycin on ULBP2, sULBP2 and their candidate shedder proteinase, a disintegrin and metalloproteinase domain 10 (ADAM10) and ADAM17,²⁰ in human cells originated from the lung.

MATERIALS AND METHODS

Cells

A549 lung cancer cell lines were obtained from the RIKEN cell bank (Tsukuba, Japan). LCSC #2 lung cancer cell lines were provided by the Cell Resource center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). A549 cells were maintained in Dulbecco's Modified Eagles Medium (Wako, Osaka, Japan) with 10% heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin and 50 units/mL streptomycin. LCSC #2 cells

Table 1. Primers used in this study

Gene	Amplicon size (bp)	Sequences of primers
ULBP2	108	Forward: ccgctaccaagatccttctg Reverse: ggatgacgggtgatgtcatagc
ADAM10	60	Forward: atattacggaacacgagaagctg Reverse: tcaatcgctttaacatgactgg
ADAM17	69	Forward: cctttctgcgagaggggaac Reverse: caccttgcaggagttgtcagt
GAPDH	66	Forward: agccacatcgctgagaca Reverse: gcccaatacgcaccaatcc

ADAM, a disintegrin and metalloproteinase domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ULBP2, UL16-binding protein 2.

were maintained in RPMI 1640 (Wako) with 10% heat-inactivated FBS, 50 units/mL penicillin and 50 units/mL streptomycin. Both cell lines were cultured in humidified air with 5% CO₂ at 37 °C.

Reagents

Clarithromycin was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tumor necrosis factor (TNF)-alpha processing inhibitor-2 (TAPI-2) was obtained from Enzo Life Science (Farmingdale, NY). Anti-ULBP2 (clone 165903) and recombinant ULBP2-Fc were obtained from R&D Systems (Minneapolis, MN). Anti-ULBP2 (BUMO1) was purchased from Bamomab (Munich, Germany) and goat anti-mouse IgG2a was purchased from SouthernBiotech Associates (Birmingham, AL). Isotype control IgG2a was obtained from Sigma-Aldrich (St. Louis, MO).

RNA extraction and cDNA synthesis

Total RNA was extracted from cells using a QIAGEN RNeasyPlus Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RNA was dissolved in 50 mL of Rnase-free water and stored at -80 °C until use in a cDNA preparation reaction. An aliquot was removed to determine the concentration of RNA in a spectrophotometer at 260 nm. For cDNA synthesis, each 20 mL reaction contained 1 mg of total RNA, as described in the QIAGEN QuantiTect Reverse Transcription Kit (Qiagen). Synthesis of cDNA was performed in a TaKaRa PCR Thermal Cycler (TaKaRa, Kyoto, Japan) according to the following procedure: the RNA samples were incubated in gDNA Wipeout Buffer (Qiagen) at 42 °C for 2 min; Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix (Qiagen) were added and incubated at 42 °C for 15 min; and the reverse transcriptase was inactivated for 3 min at 95 °C. The cDNA was stored at -30 °C until use in real-time PCR.

Quantitative real-time PCR

A LightCycler 480 (Roche Diagnostics, Mannheim, Germany) was used for all quantitative PCRs. Table 1 shows the gene-specific primers used. All PCR amplification reactions were performed with 0.5 mM forward primers, 0.5 mM reverse primers (Sigma-Genosys, Ishikari, Japan), 0.4 mM hydrolysis probes for each target gene from a universal probe library (Roche Diagnostics), 1× LightCycler 480 Probes Master (Roche

Diagnostics) and 4 mL diluted cDNA (1:10). The cycling parameters used were as follows: one denaturation cycle for 600 s at 95 °C and 45 amplification cycles (temperature transition rate of 4.4 °C s⁻¹) of 10 s at 95 °C, annealing for 25 s at 55 °C and extension for 1 s at 72 °C. Fluorescence readings were taken after each cycle following the extension step. The LightCycler 480 software generated a standard curve from the standards and determined the gene copy number in each test sample.

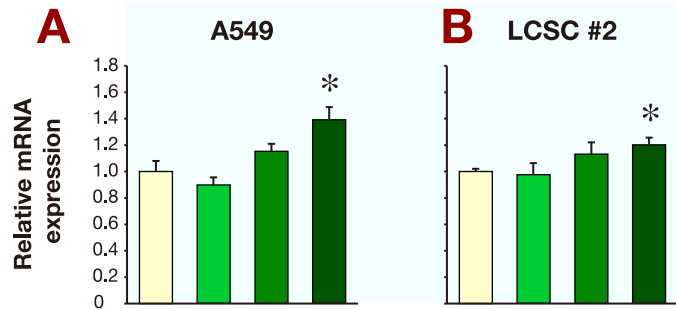
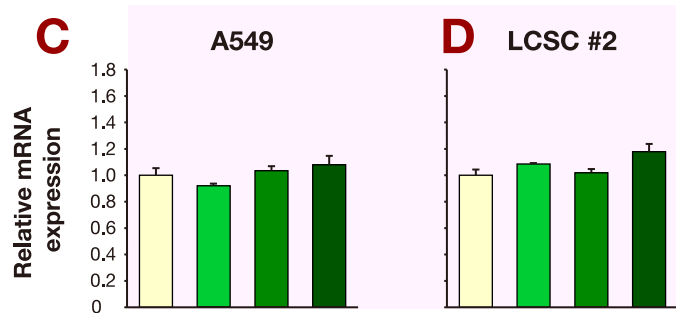
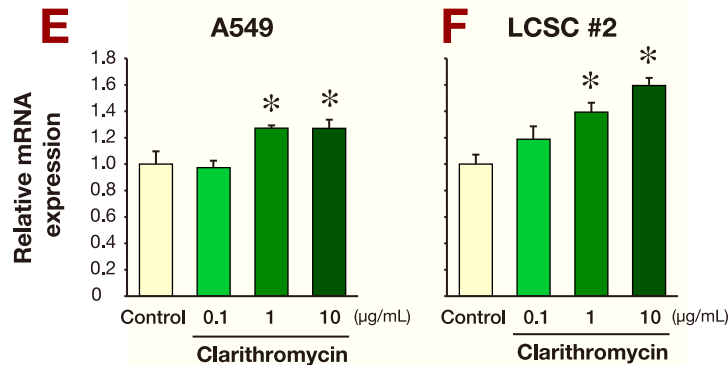
ULBP2 mRNA expression level**ADAM mRNA expression level****ADAM 10****ADAM 17**

Fig. 1. The effect of clarithromycin on the mRNA expression of ULBP2, ADAM10 and ADAM17 in A549 and LCSC #2 cell lines. A549 and LCSC #2 cells were treated with 0.1, 1 or 10 µg/mL clarithromycin for 24 h after serum starvation and harvested for total RNA extraction and quantitative real-time PCR. The ratios of gene expression between the target genes (ULBP2, ADAM10 and ADAM17) and internal standard (GAPDH) were expressed relative to those of A549 or LCSC #2 cells without clarithromycin treatment, which are set at 1.00. Data are expressed as mean values ± SD (*n* = 3 for each group). **P* < 0.05 versus control. **A:** mRNA expression of ULBP2 in A549 cells. **B:** mRNA expression of ULBP2 in LCSC #2 cells. **C:** mRNA expression of ADAM10 in A549 cells. **D:** mRNA expression of ADAM10 in LCSC #2 cells. **E:** mRNA expression of ADAM17 in A549 cells. **F:** mRNA expression of ADAM17 in LCSC #2 cells. ADAM, a disintegrin and metalloproteinase domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ULBP2, UL16-binding protein 2.

The ratios of gene expression between the target genes (ULBP2, ADAM10, and ADAM17) and the internal standard (GAPDH) were expressed relative to those of A549 or LCSC #2 without clarithromycin, which is set at 1.00.

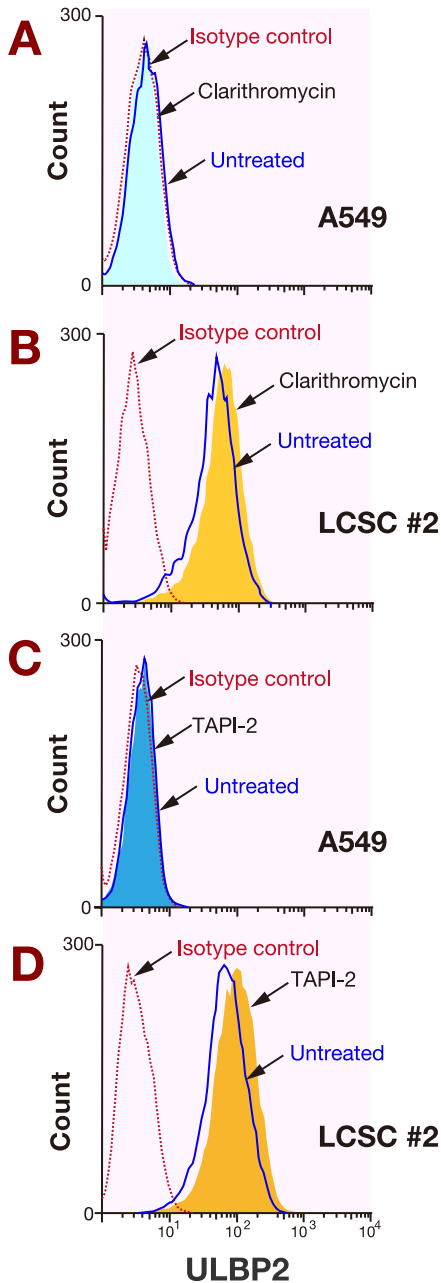


Fig. 2. The effect of clarithromycin on the cell surface expression of ULBP2 in A549 and LCSC #2 cell lines. A549 and LCSC #2 cells were treated with or without 10 μ M clarithromycin or 50 μ M TAPI-2 for 24 h after serum starvation and harvested for flow cytometry. **A:** A549 cells treated with or without 10 μ M clarithromycin. **B:** LCSC #2 cells treated with or without 10 μ M clarithromycin. **C:** A549 cells treated with or without 50 μ M TAPI-2. **D:** LCSC #2 cells treated with or without 50 μ M TAPI-2. TAPI-2, TNF-alpha processing inhibitor-2; ULBP2, UL16-binding protein 2.

Flow cytometry

Cells were incubated with monoclonal anti-ULBP2 antibodies or isotype controls. After washing, goat anti-mouse IgG labeled with FITC was added, and the fluorescence intensities of samples were analyzed on a FACSCalibur (BD Biosciences, La Jolla, CA). Data from 10,000 cells were collected, and the geometric mean fluorescence intensity (MFI) was calculated using CELLQuest software (BD Biosciences). MFI ratios were calculated by dividing the MFI obtained with a specific antibody by that for the isotype control.

ELISA

Two monoclonal anti-ULBP2 antibodies were used to detect sULBP2. Plates were coated with the anti-ULBP2 mAb BUMO1 at 2 mg/mL in coating buffer for 24 h at 4 $^{\circ}$ C, then blocked with blocking buffer for 18 h at 4 $^{\circ}$ C. The blocking buffer was prepared with a commercially available blocking agent (Applied Bioscience, Mumbai, India). Next, ULBP2-Fc (standard) and the samples were added, and the plates were incubated for 1 h at room temperature. For analysis of serum samples, ULBP2-Fc was diluted in blocking buffer with 25% horse serum, and sera were diluted 1:3 in blocking buffer before addition to the plates. After incubation, the detector mAb (anti-ULBP2, clone 165903) was added at 0.5 mg/mL in blocking buffer for 1 h at room temperature, followed by incubation with anti-mouse IgG2a-HRP (1:10,000 in blocking buffer) for 1 h at 37 $^{\circ}$ C. Color was developed with a Sumilon peroxidase ELISA kit (Sumitomo Chemical, Tokyo), and the absorbance was read at 490 nm on a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA).

TACE activity assay

The activity of ADAM17, which is also known as TACE, was determined using the SensoLyte 520 TACE Activity Assay Kit (AnaSpec, San Jose, CA) with 10 μ g of cell lysate proteins from each sample according to the manufacturer's protocol. This assay uses a 5-carboxyfluorescein (5-FAM)-labeled fluorescence resonance energy transfer (FRET) peptide substrate. Upon cleavage of the FRET peptide by the active enzyme, the fluorescence of 5-FAM is recovered and can be monitored at excitation/emission wavelengths of 490/520 nm. Fluorescence of the cleavage product was measured in a fluorescence microplate reader (TECAN, Seestrasse, Switzerland). Results are expressed as the mean change in fluorescence intensity per min.

Statistical analysis

Statistical analyses were performed using the SPSS 21.0 software (International Business Machines Corporation,

Armonk, NY) and $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of clarithromycin on ULBP2 mRNA expression

To evaluate the influence of clarithromycin on ULBP2 and its shedding mechanism, we first assessed the effect of clarithromycin on mRNA expression of ULBP2, ADAM10 and ADAM17. We used two cell lines, A549 and LCSC #2, which express ULBP2 at low and high levels, respectively. Transcript levels of ULBP2 were significantly up-regulated in A549 (Fig. 1A) and LCSC #2 (Fig. 1B) cells treated with 10 $\mu\text{g}/\text{mL}$ clarithromycin for 24 h. Additionally, although there was no significant effect of clarithromycin on the mRNA expression of ADAM10 in A549 (Fig. 1C) or LCSC #2 (Fig. 1D) cells, ADAM17 mRNA expression was up-regulated in A549 (Fig. 1E) and LCSC #2 (Fig. 1F) cells treated with 1 or

10 $\mu\text{g}/\text{mL}$ clarithromycin for 24 h. These data suggest that clarithromycin induces transcription of ULBP2 and ADAM17.

Effect of clarithromycin on cell surface and soluble ULBP2

Next, to identify the effect of clarithromycin on protein expression of ULBP2, we evaluated the changes of cell surface ULBP2 expression in A549 and LCSC #2 cells after 24 h treatment with or without 10 $\mu\text{g}/\text{mL}$ clarithromycin (Figs. 2A and B). Although there was no significant difference of cell surface ULBP2 expression in A549 cells with or without 10 $\mu\text{g}/\text{mL}$ clarithromycin treatment (Fig. 2A, MFI ratio: 1.13 versus 1.11, respectively), the expression level of ULBP2 in LCSC #2 cells with 10 $\mu\text{g}/\text{mL}$ clarithromycin was significantly higher than that without clarithromycin (Fig. 2B, MFI ratio: 22.6 versus 11.5, respectively). To test the contribution of ULBP2 shedding to this change, we next examined the effect of TAPI-2, an ADAM17 inhibitor, on the expression of ULBP2. The same tendency was observed when LCSC #2 cells were treated with 50 μM TAPI-2 for 24 h (Figs. 2C and D). Therefore, clarithromycin may mimic the effect of TAPI-2, especially for cells with abundant expression of ULBP2 such as LCSC #2 cells. These data suggest that clarithromycin induces cell surface ULBP2 expression. In addition, the mechanism of this effect is presumably induction of ULBP2 gene transcription and inhibition of ULBP2 shedding by ADAM17.

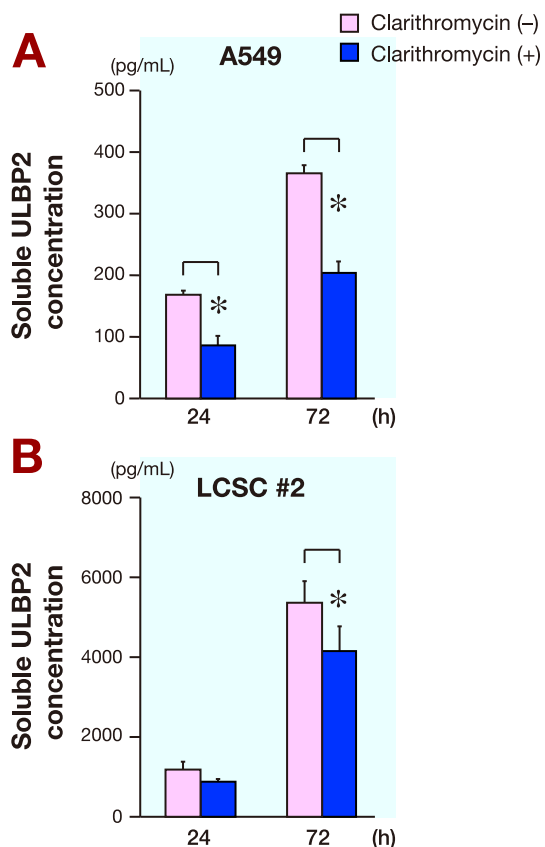


Fig. 3. The effect of clarithromycin on the level of sULBP2 in A549 and LCSC #2 cell lines. Measurement of sULBP2 in culture supernatants using ELISA. A549 and LCSC #2 cells were treated with or without 10 $\mu\text{g}/\text{mL}$ clarithromycin for 24 or 72 h after serum starvation. After treatment, the culture supernatants were harvested for ELISA. Data are expressed as mean values \pm SD ($n = 3$ for each group). * $P < 0.05$. **A:** A549 cells treated with or without 10 $\mu\text{g}/\text{mL}$ clarithromycin. **B:** LCSC #2 cells treated with or without 10 $\mu\text{g}/\text{mL}$ clarithromycin. sULBP2, solubilized ULBP2.

Quantification of sULBP2

Previously, we showed that ULBP2 is shed into the culture medium in proportion to the level of its cell surface expression by non-small cell lung cancer cell lines.¹⁹ Therefore, to reveal the effect of clarithromycin on sULBP2, we used ELISA to analyze culture supernatants from A549 (Fig. 3A) and LCSC #2 cells (Fig. 3B) at 24 and 72 h after treatment with or without clarithromycin. The number of the cells did not differ significantly under each experimental condition (data not shown); however, the amount of sULBP2 increased over time in these cell lines. The amount of sULBP2 with clarithromycin was significantly decreased relative to that without clarithromycin in both A549 and LCSC #2 cells (Figs. 3A and B). These findings suggest an inhibitory effect of clarithromycin on the ULBP2 shedding mechanism.

Evaluation of ADAM17 activity

To explore the effect of clarithromycin on the ULBP2 shedding mechanism, we next examined the effect of clarithromycin on the activity of ADAM17 because reduction of sULBP2 was observed despite increased transcription of the shedder enzyme ADAM17. As shown in

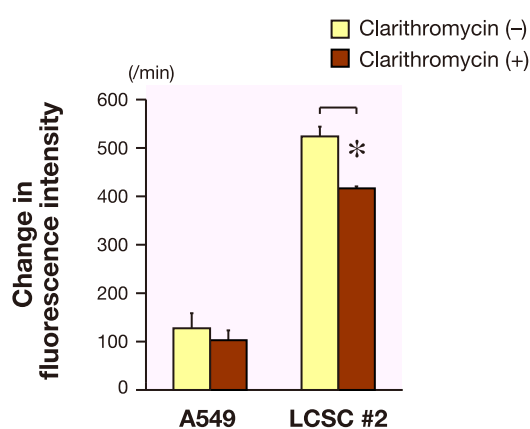


Fig. 4. The effect of clarithromycin on ADAM17 activity in A549 and LCSC #2 cell lines. ADAM17 activity was determined using the SensoLyte 520 TACE Activity Assay Kit with 10 μ g of cell lysate proteins for each sample according to the manufacturer's protocol. A549 and LCSC #2 cells were treated with or without 10 μ g/mL clarithromycin for 24 h after serum starvation and harvested for the assay. Data are expressed as the mean change in fluorescence intensity per min \pm SD ($n = 3$ for each group). * $P < 0.05$. ADAM, a disintegrin and metalloproteinase domain.

Fig. 4, 10 μ g/mL clarithromycin significantly inhibited the activity of ADAM17 in LCSC #2 cells with high cell surface ULBP2 expression. However, the effect of clarithromycin on A549 cells, which expressed lower levels of ULBP2, was not significant. This finding demonstrates that clarithromycin inhibits ADAM17 activity and, consequently, the ULBP2 shedding mechanism.

DISCUSSION

In this study, we examined the effect of clarithromycin on ULBP2 expression. Our results showed that clarithromycin induced the transcription and expression of cell surface ULBP2 while reducing the amount of sULBP2 in the culture medium. Additionally, clarithromycin inhibited the activity of ADAM17, a shedder enzyme for cell surface NKG2D ligands.²¹ Because immune effector cells such as NKT cells, CD8+T cells and NK cells are activated by enhanced expression of ULBP2 on the surfaces of target cells and are affected by only low levels of sULBP2, these pleiotropic effects of clarithromycin on ULBP2 expression might contribute to the enhanced function of immune effector cells, thereby augmenting *P. aeruginosa* clearance in the lung.

In this study, we first reported the up-regulation of ULBP2 and ADAM17 gene transcription in human cells derived from the lung. Previous studies reported that clarithromycin influences the gene expression of various proteins in human cells, including both reduction and induction of genes involved in inflammation. For example, studies have shown that clarithromycin inhibited gene

transcription of mucin,²² IL-8²³ and various cytokines such as TNF-alpha, interferon gamma, interleukin (IL)-2, IL-4, IL-5 and IL-6.²⁴ On the other hand, transcription of IL-10²⁵ and alpha1-acid glycoprotein was induced by clarithromycin in hepatocytes.²⁶ Toward understanding the mechanism of gene transcription modulation by clarithromycin, a previous study reported that clarithromycin inhibits activator protein-1 transcription factor²³ or potentiates glucocorticoid response element.²⁶ The precise mechanism leading to up-regulation of ULBP2 and ADAM17 is still unclear, but it may occur through the modulation of certain transcription factors. Because most previous reports focused on the effect of clarithromycin on the inflammatory cytokines, its effect on other proteins involved in the immune process and its mechanism of action must be explored in future studies.

In addition to its role in transcriptional regulation, we revealed that clarithromycin inhibits the enzymatic activity of ADAM17. The inhibitory effect of clarithromycin on enzyme activity has been reported in other enzymes such as cytochrome P450 3A,²⁷ human serum paraoxonase²⁸ and glycosidase.²⁹ This effect of clarithromycin on ADAM17 activity represents a new mechanism that may explain the recent reports of broad pleiotropic activities of macrolide antibiotics for immunomodulatory,³⁰ anti-inflammatory³¹ and antitumor³² effects. Previous studies revealed that ADAM17, originally identified as an enzyme that proteolytically cleaves pro-TNF-alpha to generate soluble TNF-alpha,³³ cleaves and solubilizes a wide range of membrane-bound proteins including transforming growth factor (TGF)-alpha³⁴ and heparin-binding epidermal growth factor-like growth factor (HB-EGF).³⁵ The substrate proteins of ADAM17 make obvious contributions to various pathogenesises, for example, TNF-alpha is involved in inflammation, and both TGF-alpha and HB-EGF contribute to tumorigenesis. Therefore, inhibition of the activity of ADAM17 by clarithromycin might represent a novel mechanism for its effect on these pathological conditions.

Recent reports suggested that *P. aeruginosa* infection induced the surface expression of human NKG2D ligands such as ULBP2 in airway epithelial cells and signified the indispensability of induced NKG2D-mediated immune activation in the clearance of this bacterium.^{16, 18} In addition, we previously revealed that *P. aeruginosa* is actively involved in this process by secreting a quorum sensing molecule, N-3-oxo-dodecanoyl homoserine lactone, that decreases cell surface ULBP2 and increases sULBP2.³⁶ This intervention by *P. aeruginosa* leads to attenuation of the host NKG2D-mediated immune response and is beneficial for its survival in the human lung. Our novel finding in this study that

clarithromycin increases the cell surface ULBP2 expression and decreases sULBP2 suggests a mechanism by which it counteracts the immunosuppressive activity of *P. aeruginosa* and might explain the effectiveness of clarithromycin in treating chronic *P. aeruginosa* infection in chronic lung diseases.³⁷

In conclusion, we revealed that clarithromycin induces expression of ULBP2 and, through ADAMI7, reduces sULBP2. These findings suggest the possibilities that clarithromycin can activate NKT cells and decrease the colonization of bacteria in patients with chronic respiratory diseases such as diffuse panbronchiolitis. Therefore, further study toward augmenting the effect of clarithromycin on NKT cell activity through ULBP2 might be useful for developing new macrolide-based treatments for chronic respiratory diseases.

The authors declare no conflict of interest.

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