Different effects of telithromycin on MUC5AC production induced by human neutrophil peptide-1 or lipopolysaccharide in NCI-H292 cells compared with azithromycin and clarithromycin

Hiroshi Ishimoto1, Hiroshi Mukae1*, Noriho Sakamoto1, Misato Amenomori1, Takeshi Kitazaki1, Yoshifumi Imamura1, Hanako Fujita1, Hiroshi Ishii2, Seiko Nakayama1, Katsunori Yanagihara3 and Shigeru Kohno1

1Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan; 2Department of Infectious Diseases, Oita University Faculty of Medicine, Oita, Japan; 3Department of Laboratory Medicine, Nagasaki University School of Medicine, Nagasaki, Japan

Received 23 April 2008; returned 3 July 2008; revised 7 September 2008; accepted 18 September 2008

Objectives: Mucus hypersecretion is a prominent feature in patients with chronic respiratory tract infections such as cystic fibrosis and diffuse panbronchiolitis, and the clinical effectiveness of macrolide antibiotics has been reported in these patients. Because human neutrophil peptide-1 (HNP-1), an antimicrobial peptide in neutrophils, exists in high concentrations in the airway fluid of these patients, we examined the direct effect of HNP-1 on MUC5AC mucin production using NCI-H292 cells. The effects of macrolide antibiotics on the response were also examined.

Methods: MUC5AC synthesis was assayed using RT–PCR and ELISA. Phosphorylation of ERK1/2 was determined by western blotting.

Results: Stimulation with HNP-1 or lipopolysaccharide (LPS) derived from Pseudomonas aeruginosa increases the production of MUC5AC mRNA and protein, and an additive effect was found upon co-stimulation with both HNP-1 and LPS. Azithromycin and clarithromycin had inhibitory effects on overproduction of MUC5AC induced by HNP-1 or LPS stimulation. Telithromycin also had an inhibitory effect on MUC5AC production induced by LPS, but not on production by HNP-1. Phosphorylation of ERK1/2 was induced by HNP-1 or LPS stimulation, and azithromycin, clarithromycin and telithromycin had inhibitory effects on ERK1/2 phosphorylation induced by LPS, but not by HNP-1.

Conclusions: These findings suggest that neutrophil-derived defensins as bacterial components contribute to excessive mucus production in patients with respiratory tract infections, and that macrolide and ketolide antibiotics directly inhibit these actions by interfering with intracellular signal transduction. However, the mechanism of telithromycin inhibition of MUC5AC synthesis may differ from the response induced by azithromycin and clarithromycin.

Keywords: HNP-1, LPS, macrolide antibiotics, ERK1/2 phosphorylation

Introduction

In the respiratory tract, mucus secretion is useful for host protection against pathogens and irritants, and the mucus layer assists in clearance of inhaled foreign materials. However, the overproduction of sputum is one of the clinical features of chronic airway diseases including chronic bronchitis, bronchiectasis, cystic fibrosis (CF) and diffuse panbronchiolitis (DPB). In these diseases, mucus hypersecretion is an important hallmark of pathogenesis because it causes airway obstruction and impairment of gas exchange. Thus, preventing mucus overproduction is suggested to be beneficial in chronic airway diseases. The major macromolecular component of mucus is the mucin protein encoded by MUC genes. Of the 19 currently identified human MUC genes, MUC5AC is one of the major respiratory mucins secreted from the airway surface epithelium.
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Among the many factors that contribute to mucin hypersecretion, chronic bacterial infection is considered to be the most important one in respiratory diseases such as DPB and CF. In particular, chronic infection of *Pseudomonas aeruginosa* in the lower respiratory tract often causes trouble during the clinical course of these diseases. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria including *P. aeruginosa*, can evoke extreme biological responses from the host, including fever, procoagulant activity, septic shock and death.\(^3\) In addition, *P. aeruginosa* and its LPS induce MUC5AC production in airway epithelial cells *in vitro*.\(^5\) These findings suggest the importance of bacterial products to hypersecretion in respiratory tract infections.

Human neutrophil peptide-1 (HNP-1), which is one of the α-defensins and is mainly present in the azurophilic granules of neutrophils, has strong antimicrobial activity.\(^4\) HNP-1 also has chemotactic activity for monocytes, T cells and dendritic cells,\(^4\) and stimulates interleukin (IL)-8 production\(^5\) and biofilm formation.\(^6\)

The effects of macrolide antibiotics are thought to modulate neutrophil functions,\(^11\) IL-8 production\(^5\) and biofilm formation produced by *P. aeruginosa*.\(^12\) We also reported previously that macrolides inhibit overexpression of MUC5AC in a murine model of DPB\(^13\) and patients with DPB.\(^14\) These findings suggest that macrolides may also inhibit the HNP-1-induced overproduction of MAC5AC in bronchial epithelial cells. Telithromycin, a novel class of antibacterial agents structurally related to the macrolides (ketolide antibiotic), is active against erythromycin-resistant pneumococci.\(^15\) Recently, the TELICAST study showed evidence of a benefit of telithromycin to patients with acute exacerbation of asthma.\(^16\) Although the mechanisms of this benefit remain unclear, it is possible that telithromycin also has immunomodulatory effects similar to the macrolides\(^8\) and clarithromycin both *in vitro*\(^17\) and *in vivo*.\(^18\)

The aims of this study were to determine the co-stimulatory action of external pathogenic agent LPS and internal antimicrobial agent HNP-1 on the production of MUC5AC, and to clarify whether telithromycin has a different effect on MUC5AC production induced by LPS and HNP-1 compared with azithromycin and clarithromycin.

**Materials and methods**

**Materials**

Synthetic HNP-1 peptide was obtained from the Peptide Institute (Osaka, Japan). LPS derived from *P. aeruginosa* (lot 87F4009) was obtained from Sigma (St Louis, MO, USA). Azithromycin, clarithromycin and telithromycin were, respectively, provided by Pfizer (Tokyo, Japan), Taisho-Toyama (Tokyo, Japan) and Sanofi Aventis (Paris, France). Mouse monoclonal MUC5AC antibody (clone 45M1) was obtained from Neo Markers (Fremont, CA, USA). ERK1/2 and phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). PD98059, an extracellular signal-regulated kinase (ERK) pathway inhibitor, was obtained from Promega (Madison, WI, USA).

**Cell culture**

NCI-H292 airway epithelial cell lines were obtained from the ATCC (Manassas, VA, USA). The cells were grown at 37 °C in 5% CO₂ fully humidified air in RPMI 1640, supplemented with 10% fetal bovine serum and 100 mg/L penicillin–streptomycin. The cells were seeded in a 6-well plate at 5 × 10⁵ cells/well. In the MUC5AC production studies, cells were then exposed to 1–50 mg/L HNP-1 or 100 pg/mL to 1 mg/L LPS, for 12 h for RT–PCR or 24 h for ELISA. In the inhibition studies, cells were pre-treated with 10 mg/L azithromycin, clarithromycin or telithromycin for 6 h before exposure to 10 mg/L HNP-1 or 10 ng/mL LPS, and cells were pre-treated with 10 μM PD98059 for 2 h before exposure to 10 mg/L HNP-1. In the case of controls, the cells were incubated with medium alone.

**RT–PCR**

The NCI-H292 cells were cultured, harvested and RNA was extracted with ISOGEN (Nippon Gene, Toyama, Japan). Oligonucleotide primers for PCR were designed according to the published sequence for human MUC5AC (sense: ATC ACC GAA GGC TGC TTC TGT C; antisense: GTT GAT GCT GCA CAC TGT CCA G). PCR products were separated by electrophoresis through 1% agarose gel containing ethidium bromide, and the signal intensity was analysed by NIH Image. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls were used to standardize the quantification of RNA samples.

**ELISA**

The NCI-H292 cells were plated in a 6-well plate, and the MUC5AC protein was measured by ELISA according to a procedure described previously.\(^19\) Cell lysates were incubated at 40 °C in a 96-well plate until dry. Plates were blocked with 2% bovine serum albumin for 1 h at room temperature and incubated with MUC5AC antibody diluted with PBS containing 0.05% Tween 20 for 1 h. Horseradish peroxidase (HRP)-conjugated anti-goat IgG was dispensed into each well. The plates were washed three times with PBS. After 4 h, the plates were washed three times with PBS. Colour was developed using a 3,3′,5,5′-tetramethylbenzidine peroxidase solution and stopped with 2 N H₂SO₄. Absorbance was read at 450/620 nm. Because the amount of MUC5AC produced by the NCI-H292 cells varied depending on the passage number of the cells used,\(^20\) the percentage above the control value was used to represent the MUC5AC data.

**Western blot analysis**

Proteins were separated using 12% reducing SDS–PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 20% methanol/25 mM Tris–HCl/0.2 M glycine. Non-specific binding was blocked by incubating the membranes with 5% skimmed milk in tris buffer saline (TBS) with 0.1% Tween 20 for 1 h at room temperature. Immunoreactive proteins were detected by incubating the membranes with rabbit anti-human ERK1/2 and phospho-ERK1/2 antibodies (each 1:1000) overnight at 4°C. Subsequently, the membranes were incubated for 1 h with...
anti-rabbit IgG conjugated to HRP (1:10 000), re-washed and
developed using enhanced chemiluminescence (ECL) reagents
(Amersham Pharmacia Biotech). Western blot images were scanned
and analysed using NIH Image J software.

Statistical analysis
All data are expressed as means ± SEM. Differences were exam-
ined for statistical significance using one-way analysis of variance.
The post hoc test was Fisher’s protected least significant difference
(PLSD) test. A P value < 0.05 denoted the presence of a statistically
significant difference.

Results
HNP-1 and LPS up-regulate MUC5AC gene and protein
expression
To confirm whether HNP-1 and LPS can induce mucin production
in NCI-H292 cells, we first evaluated the MUC5AC expression at
both the mRNA and protein levels after addition of HNP-1 or LPS
to the cells. We found that HNP-1 activated the cells to express
MUC5AC and that both MUC5AC mRNA and protein levels were
maximal at 10 mg/L HNP-1 (Figure 1a and b). We also examined
the time-dependent effect of HNP-1 on MUC5AC synthesis. The
levels of MUC5AC mRNA and protein increased significantly at
12 and 24 h, respectively, after the addition of HNP-1 (data not
shown). The mRNA and protein expression of MUC5AC also
increased as a result of LPS stimulation in a concentration-
dependent manner (Figure 2a and b).

In CF and DPB, both defensins and LPS derived from
P. aeruginosa are present abundantly in the bronchial epithelial
lining fluid, so simultaneous stimulation with HNP-1 and LPS
was performed to determine whether an additive effect was
present. Stimulation with low-dose HNP-1 and low-dose LPS
did not affect MUC5AC production, but concurrent stimulation
with 10 mg/L HNP-1 and 10 ng/mL LPS indicated an additive
effect on MUC5AC production in NCI-H292 cells at both the
mRNA and protein levels (Figure 3a and b).

Macrolides inhibit MUC5AC production induced by HNP-1

Figure 1. Up-regulation of MUC5AC induced by HNP-1. NCI-H292 cells
were stimulated with 1–50 mg/L HNP-1. MUC5AC mRNA levels after 12 h
of incubation (a) and MUC5AC protein levels after 24 h of incubation
(b). Data represent means ± SEM of four experiments. *P < 0.05 compared
with culture medium alone.

Figure 2. Concentration-dependent effect of LPS on MUC5AC synthesis.
NCI-H292 cells were stimulated with 100 pg/mL to 1 mg/L LPS. MUC5AC
mRNA levels after 12 h of incubation (a) and MUC5AC protein levels after
24 h of incubation (b). Data represent means ± SEM of four experiments.
*P < 0.05 compared with culture medium alone.

Figure 3. Additive effect of HNP-1 and LPS on MUC5AC synthesis.
NCI-H292 cells were stimulated with a combination of 1 mg/L HNP-1 and
1 ng/mL LPS, or 10 mg/L HNP-1 and 10 ng/mL LPS. MUC5AC mRNA
levels after 12 h of incubation (a) and MUC5AC protein levels after 24 h of
incubation (b). Data represent means ± SEM of four experiments. *P < 0.05
compared with culture medium alone.
Effect of azithromycin, clarithromycin and telithromycin on MUC5AC production of NCI-H292 cells stimulated with HNP-1 and LPS

Certain macrolide antibiotics have been reported to reduce mucus hypersecretion in vivo and in vitro,3,13,14 thus we next evaluated the effects of azithromycin and clarithromycin on MUC5AC expression induced by HNP-1 or LPS. We also evaluated the effect of one of the ketolide antibiotics, telithromycin. The cells were pre-treated with azithromycin, clarithromycin and telithromycin at 10 mg/L each for 6 h before the addition of HNP-1 or LPS. Azithromycin and clarithromycin significantly reduced the enhanced expression of MUC5AC induced by HNP-1 at both the mRNA and protein levels (Figure 4a and b). In contrast, telithromycin did not reduce the MUC5AC expression induced by HNP-1 (Figure 4a and b). However, azithromycin, clarithromycin and telithromycin all reduced the elevated expression of MUC5AC induced by LPS stimulation at the mRNA and protein levels (Figure 5a and b).

Increased ERK1/2 phosphorylation in HNP-1- and LPS-activated cells

To ascertain the participation of ERK1/2 phosphorylation in MUC5AC production induced by HNP-1 stimulation, we treated the cells with 10 μM PD98059 for 2 h before the addition of 10 mg/L HNP-1. An inhibitor of MEK (PD98059) reduced the MUC5AC expression induced by HNP-1 stimulation at the mRNA level (data not shown).

To study the involvement of ERK1/2 activation in MUC5AC production induced by HNP-1 and LPS stimulation in NCI-H292 cells, we treated the cells with HNP-1 or LPS for 15 min and evaluated ERK1/2 phosphorylation by western blotting. The phosphorylation of ERK1/2 was stimulated in HNP-1- or LPS-treated cells, but not in untreated cells (Figure 6a and b). To better understand the efficacy of macrolide or ketolide antibiotics on MUC5AC production, we also examined the influence of macrolide and ketolide antibiotics on ERK1/2 phosphorylation. When the cells were pre-treated with azithromycin, clarithromycin or telithromycin, the phosphorylated forms of the ERK1/2 kinases induced by LPS were decreased (Figure 6b). However, the phosphorylated forms of the ERK1/2 kinases induced by HNP-1 did not decrease by pre-treatment with azithromycin, clarithromycin or telithromycin (Figure 6a).

Discussion

The present study is the first to demonstrate that HNP-1 induces not only MUC5AC mRNA expression but also protein production, and that an additive effect of HNP-1 and LPS on MUC5AC in airway epithelial cells exists. We also showed that a ketolide antibiotic, telithromycin, inhibits MUC5AC production induced by LPS, but not by HNP-1, while clarithromycin and azithromycin inhibit both LPS- and HNP-1-induced MUC5AC production. Furthermore, clarithromycin, azithromycin and telithromycin reduce phosphorylation of ERK1/2 induced by LPS, but not by HNP-1.

Excessive mucus secretion is problematic in patients with chronic airway diseases due to airway obstruction and impairment of gas exchange. In addition, a recent report also

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**Figure 4.** Effect of azithromycin (AZM), clarithromycin (CLR) and telithromycin (TEL) on MUC5AC production induced by HNP-1 stimulation. NCI-H292 cells were stimulated with 10 mg/L HNP-1 after pre-treatment with or without azithromycin, clarithromycin or telithromycin for 6 h. MUC5AC mRNA levels after 12 h of incubation (a) and MUC5AC protein levels after 24 h of incubation (b). Data represent means ± SEM of four experiments. *P < 0.05 compared with 10 mg/L HNP-1 stimulation alone.

**Figure 5.** Effects of azithromycin (AZM), clarithromycin (CLR) and telithromycin (TEL) on MUC5AC production induced by LPS stimulation. NCI-H292 cells were stimulated with 10 ng/mL LPS after pre-treatment with or without azithromycin, clarithromycin or telithromycin for 6 h. MUC5AC mRNA levels after 12 h of incubation (a) and MUC5AC protein levels after 24 h of incubation (b). Data represent means ± SEM of four experiments. *P < 0.05 compared with 10 ng/mL LPS stimulation alone.
demonstrated that the in vitro activity of human β-defensin 2, an antimicrobial peptide such as HNP-1, against P. aeruginosa was reduced in the presence of MUC5AC. This suggests that mucin hypersecretion may affect the bactericidal activity of various antimicrobial proteins and polypeptides existing in the airway fluid, leading to a predisposition to infections in these diseases. Therefore, it is important to control mucus secretion in these patients.

LPS is a major component of the outer membrane of Gram-negative bacteria including P. aeruginosa, which is a major pathogenic agent in patients with chronic respiratory tract infection. LPS is well known to induce MUC5AC production in airway epithelial cells and is considered to play a pivotal role in chronic respiratory tract infection. The levels of HNP-1, one of the α-defensins mainly present in neutrophils, are also increased in focal lesions of patients with chronic respiratory infectious diseases such as CF and DPB. HNP-1 was originally considered an antimicrobial peptide, but recent studies have demonstrated that defensins including HNP-1 act as immune regulatory factors. In the present study, we demonstrated that internal non-pathogenic agent HNP-1 and external pathogenic agent LPS have additive effects on mucin production in airway epithelial cells. This suggests that high concentrations of bacterial products and defensins in the epithelial lining fluid may cause hypersecretion in the associated diseases. In addition, a high dose of HNP-1 also has cytotoxic effects against host cells including bronchial epithelial cells and lung fibroblasts. Therefore, HNP-1 may play a negative role, in addition to LPS, in the pathogenesis of chronic respiratory tract infection.

In this study, we also demonstrated that telithromycin reduces MUC5AC production induced by LPS. Long-term treatment with macrolide antibiotics is considered to be effective in DPB and CF due to anti-inflammatory effects rather than antimicrobial effects. In addition, a recent report by Tahan et al. showed that clarithromycin treatment might be helpful in reducing the short-term effects of respiratory syncytial virus bronchiolitis. However, the existence of anti-inflammatory effects of telithromycin similar to those of macrolide antibiotics remains uncertain. In this context, a recent study showed that telithromycin improved the clinical symptoms in acute exacerbations of asthma. Although the mechanism is unclear, we speculate that this efficacy of telithromycin is due to an anti-inflammatory effect, not an antimicrobial effect. A recent study demonstrated that telithromycin inhibited the secretion of IL-1α and TNF-α by monocytes, supporting this hypothesis. However, we recently reported that telithromycin significantly reduced the number of viable bacteria, but had no effect on the proliferation of lymphocytes in an experimental murine model of chronic respiratory infection in vivo. In contrast, clarithromycin decreased the number of lymphocytes, but had no effect on the number of viable bacteria in the lung. This suggests that telithromycin and clarithromycin have different effects on chronic respiratory infections caused by P. aeruginosa.

Figure 6. Effect of HNP-1 (a) and LPS (b) on the phosphorylation of ERK1/2 and the effect of azithromycin (AZM), clarithromycin (CLR) and telithromycin (TEL) on the phosphorylated ERK (p-ERK)/t-ERK induced by HNP-1 and LPS. NCI-H292 cells were stimulated with 10 mg/L HNP-1 or 10 ng/mL LPS after pre-treatment with or without azithromycin, clarithromycin or telithromycin for 6 h. p-ERK was evaluated by western blotting. Data represent means ± SEM of four experiments. *P < 0.05 compared with culture medium alone or 10 ng/mL LPS stimulation alone. The band intensity of p-ERK and total ERK (t-ERK) was calculated using NIH Image J.

Acknowledgements
We thank N. Araki for her excellent technical assistance.

Funding
This study was supported in part by Grants-in-Aid for Scientific Research (C) (KAKENHI), Japan.
Transparency declarations

None to declare.

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