Title

Immunomodulatory effect of linezolid on MRSA supernatant-induced MUC5AC overexpression in human airway epithelial cells

Running title

Effect of linezolid on MUC5AC overexpression

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Abstract

Linezolid is the first member of the oxazolidinones and is active against drug-resistant gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, linezolid showed an immunomodulatory effect, such as an inhibition of the inflammatory cytokines production. In this study, we examined the effect of linezolid on MRSA-induced MUC5AC overexpression in airway epithelial cells. In this study, an MRSA supernatant was used to avoid the direct effect of linezolid on MRSA. MUC5AC protein production was significantly increased with a 40-fold dilution of MRSA supernatant. At the messenger RNA (mRNA) level, MUC5AC gene expression was significantly increased at 6 and 9 hours after stimulation. In an inhibition study, linezolid significantly reduced MRSA-induced MUC5AC protein and mRNA overexpression at concentrations of 5 and 20 μg/mL, which were same as the trough and peak concentrations in human epithelial lining fluid. In an analysis of cell signaling, among the mitogen-activated protein kinase inhibitors, only the extracellular signal–regulated protein kinase (ERK1/2) inhibitor reduced the MUC5AC protein production to the same level as that of the control; on Western blot analysis, only ERK1/2 was phosphorylated by the MRSA supernatant. In addition, the ERK1/2 phosphorylation was inhibited by linezolid. MUC5AC as well as MUC5B is the major barrier that traps inhaled microbial organisms, particulates, and foreign irritants. However, in patients with chronic respiratory diseases, pathogen-induced MUC5AC overexpression causes many problems, and control of the overexpression is important. Thus, this study revealed that linezolid showed the direct immunomodulatory effect in airway epithelial cells.

Key words: linezolid, MRSA, mucin, MUC5AC, airway epithelial cell

Introduction

Linezolid is the first member of the oxazolidinones, a new class of antimicrobial agents. It acts by inhibiting the initiation of bacterial protein biosynthesis and is active against drug-resistant gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci, and it is one of the recommended antibiotics for patients with MRSA pneumonia in the guidelines for the management of hospital-acquired pneumonia (1) Linezolid also reduced a production of bacterial toxin (2-4). In addition, linezolid has been shown the direct immunomodulatory effects on inflammatory cells to inhibit the production of inflammatory cytokines such as interleukin (IL)-1 beta, IL-6, IL-8, and tumor necrosis factor alpha (5-7). The direct immunomodulatory effects of antimicrobial agents have been well elucidated with macrolides. Macrolides affect host cells by down-regulating inflammation, reducing production of reactive oxygen, inhibiting neutrophil activation and mobilization, accelerating neutrophil apoptosis, and blocking activation of nuclear transcription factors (8). Then, one of the immunomodulatory effects of the macrolides on human airway epithelial cells is the inhibition of pathogen-induced MUC5AC overexpression (9-14).

MUC5AC and MUC5B are gel-forming mucins that are strongly expressed in the lung (15). The Mucin is the major barrier that traps inhaled microbial organisms, particulates, and foreign irritants in airway epithelium. In particular, MUC5B may play important roles in an airway defense, because loss of *Muc5b* gene reduced survival by causing bacterial infection in a murine model (16). In contrast,, MUC5AC overexpression was observed in patients with chronic respiratory diseases such as, diffuse panbronchiolitis and asthma (17), (18). The overexpression was also observed in patients with ventilator-associated pneumonia (VAP) (19). Since mucin overexpression causes airway obstruction, atelectasis, reduction of

oxygenation, and reduction of antibiotic permeability, inhibition of MUC5AC overexpression seemed to be useful.

MUC5AC overexpression is induced by various pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Haemophilus influenzae*, *Fusobacterium nucleatum*, *Chlamydophila pneumoniae*, *and Legionella pneumophila* (9-14, 20, 21). MUC5AC overexpression also induced by peptidoglycan from *S. aureus* was reported (22). Although *S. aureus*, especially MRSA, is an important pathogen in patients with chronic respiratory diseases and VAP, there has been no study that has reported the effect of anti-MRSA antibiotics on MUC5AC overexpression. In addition, there was no report about direct immunomodulatory effect of linezolid on human airway epithelial cells. The purpose of this study was to reveal the direct immunomodulatory effect of linezolid by inhibition of MRSA-induced MUC5AC overexpression.

Materials and Methods

Materials

Linezolid (Pfizer, Groton, CT, USA) was dissolved in distilled water. The mouse anti-MUC5AC monoclonal antibody (clone 45M1) was purchased from MONOSAN (Uden, the Netherlands). The goat anti-mouse horseradish peroxidase–conjugated secondary antibody was obtained from Bio-Rad (Hercules, CA, USA). The extracellular signal–regulated protein kinase (ERK) inhibitor (U0126) was purchased from Promega (Madison, WI, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) and c-Jun N-terminal kinase (JNK) inhibitor II were purchased from Calbiochem (San Diego, CA, USA). The anti-ERK, anti–phospho-ERK1/2, anti-p38 MAPK, anti–phospho-p38 MAPK, anti-JNK, anti–phospho-JNK, anti-I-kappa B alpha (IkBa), and anti-phospho-IkBa antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The ERK1/2 control cell extracts, p38 MAPK control cell extracts, JNK control cell extracts, and NF-kappa B (NF-kB) control cell extracts also purchased from Cell Signaling Technology (Danvers, MA, USA).

Bacterial strain

The MRSA strain used in this study was NUMR101, which was a clinical isolate obtained from the blood sample of a patient at the Nagasaki University Hospital (23). The bacteria were stored at −80ºC in a Microbank bead-based preservation system (Pro-Lab Diagnostics, Ontario, CA, USA) until use. The genetic characteristic of NUMR101 was identified by real-time polymerase chain reaction (PCR) using the same method as described in a previous report (24); the staphylococcal cassette chromosome *mec* (SCC*mec*) was type II, and the strain carried virulence genes such as *sec* and *tsst* but did not carry *etb* and *pvl* genes.

Preparation of MRSA supernatant

To avoid the direct effect of linezolid on MRSA, we used an MRSA supernatant. The MRSA supernatant was prepared using a method modified described in our previous report (12). The NUMR101 strain was cultured on Mueller Hinton II agar (Becton, Dickinson and Company, Sparks, MD, USA) at 37ºC with 5% CO_2 in fully humidified air. After overnight incubation, the NUMR101 strain was harvested and incubated in 10 mL of Luria-Bertani (LB) broth (MO BIO Laboratories, Carlsbad, CA, USA) at 37ºC with shaking at 250 rpm for 72 hours. After incubation, the bacteria were centrifuged at 10,000*g* for 10 minutes at 4ºC, and the supernatant was filtered using a 0.22-μm Millex-GP filter (Millipore Corporation, Billerica, MA, USA). The MRSA supernatant was stored at −80ºC until use.

Cell Culture

The NCI-H292 (human airway epithelial) cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were grown at 37ºC with 5% CO_2 in fully humidified air. For the MUC5AC production studies, the cells were cultured in RPMI 1640 medium supplemented without FBS for 24 hours. After serum starvation, the cells were stimulated with the MRSA supernatant for enzyme-linked immunosorbent assay (ELISA) or reverse-transcription (RT)-PCR. The cells were treated with linezolid simultaneously with stimulation using the MRSA supernatant in the inhibition studies. In reference to the previous study, which reported the trough and peak concentrations of linezolid in the epithelial lining fluid (ELF) of patients with VAP (25), linezolid was used at 2 concentrations (5 and 20 μg/mL). Since there is possibility that LB broth induce MUC5AC overexpression, the controls were incubated with the same amount of LB broth as the MRSA supernatant. The cells were also pretreated with signal transduction inhibitors at

concentrations of 10 μM for 30 minutes before stimulation and the cells in the controls were incubated with only the medium and the same amount of dimethyl sulfoxide as the inhibitors.

ELISA

The MUC5AC protein level was measured using an enzyme-linked immunosorbent assay (ELISA) (10). The cells were cultured in 24-well plates, and after stimulation with the MRSA extracts for 24 hours, the culture medium was collected as the cell supernatant. The supernatant was then incubated at 40°C in a 96-well plate until dry. After incubation, the plates were blocked with 2% FBS for 1 hour at room temperature and then incubated with the anti-MUC5AC antibody diluted in phosphate-buffered saline containing 0.05% Tween 20 for 1 hour. Horseradish peroxidase–conjugated anti-goat immunoglobulin G was then dispensed into each well. After 1 hour, the color developed using a 3,3′,5,5′-tetramethylbenzidine peroxidase solution, and the reaction was stopped by the addition of 1-N H_2SO_4. The absorbance was measured at 450 nm.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the NCI-H292 cells cultured in 6-well plates using QuickGene Mini-80 and QuickGene RNA cultured cell kits (Kurabo Industries, Osaka, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into complementary DNA using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and then treated with RNase H. To quantify the expression of the MUC5AC gene, PCR primers and TaqMan probes were designed and used as reported previously (forward primer, 5′-CAGCCACGTCCCCTTCAATA-3′; reverse primer, 5′-ACCGCATTTGGGCATCC-3′; TaqMan probe, 5′-6-FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA-3′) (11). The MUC5AC was amplified for 40 cycles (15 s at 95°C and 30 s at 60°C) using a LightCycler system (Rohce

Diagnostics, Basel, Switzerland). To normalize the MUC5AC expression, human porphobilinogen deaminase was also measured using specific PCR primers and TaqMan probes (forward primer, 5′-AACCAGCTCCCTGCGAAGA-3′; reverse primer, 5′-CCAGGATGATGGCACTGAACT-3′; TaqMan probe, 5′-FAM-ACTCCTGAACTCCAGATGCGGGAACT-TAMRA-3′) (26).

Western blot analysis

The cells were harvested at 0, 30, 60, and 90 min after MRSA stimulation and then washed and homogenized at 4ºC in lysis buffer (0.1% sodium dodecyl sulfate, 1% Igepal CA-630, and 0.5% sodium deoxycholate). The cell lysates (20–50 μg) were resolved by electrophoresis on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking the membrane using 10% FBS and 0.1% Tween 20 in Tris-buffered saline for 1 hour at room temperature, the blots were hybridized overnight at 4ºC with primary antibodies. Hybridization with secondary antibodies was performed and the immunocomplexes were visualized using an ECL Western blotting detection reagent (GE Healthcare, Chalfont St. Giles, United Kingdom).

Statistical analysis

A statistical software package (StatMate IV for Windows; ATMS Co., Ltd., Tokyo, Japan) was used for all statistical comparisons. All data are expressed as mean and standard deviation (SD). One-way analysis of variance was used to determine the statistically significant differences between the groups. The Tukey test was used for pairwise comparison. All tests of significance were 2 tailed. The alpha level for denoting statistical significance was set at < 0.05.

Results

MRSA supernatant-induced MUC5AC protein production and gene overexpression

Of the several concentrations of MRSA supernatant, only the 1/40-fold dilute solution of MRSA supernatant significantly increased the protein level of MUC5AC after 24 hours of stimulation (334.1 ± 150.3% greater than control, $P < 0.05$ vs. control) (Fig. 1A). To make sure that 72 hours incubation in the preparation of MURSA supernatant was most increased protein level of MUC5AC, 6 hours (mid-log phase) and 72 hours incubation were compared. The protein level of MUC5AC was 6.6 times higher in MRSA supernatant obtained from 72 hours incubation than that from 6 hours incubation. Based on this result, the 1/40-fold dilute solution of MRSA supernatant obtained from 72 hours incubation was used for further studies. Subsequently, the influence of the MRSA supernatant on MUC5AC messenger RNA (mRNA) expression was evaluated. The cells were stimulated for 3, 6, and 9 hours, and the mRNA level of MUC5AC was assayed by RT-PCR. The MRSA supernatant significantly increased the mRNA level at 6 and 9 hours to approximately 4 times than that of the control ($P < 0.01$) (Fig. 1B).

Linezolid inhibited MRSA supernatant-induced MUC5AC protein production and gene expression

We then examined the effect of linezolid on MRSA supernatant-induced MUC5AC protein production and gene expression. Compared with the stimulation group, linezolid significantly reduced MRSA supernatant-induced MUC5AC protein production at concentrations of 5 and 20 μg/mL (373.6 ± 77.8% greater than control vs. 14.0 ± 68.8% greater than control [$P < 0.001$] and 373.6 ± 77.8% greater than control vs. 12.8 ± 13.8% greater than control [$P < 0.001$], respectively). However, this did not occur in a dose-dependent manner. At the mRNA level, linezolid significantly reduced MRSA

supernatant-induced MUC5AC mRNA expression at both concentrations compared with the stimulation group (16.1 ± 8.2 vs. 6.9 ± 1.8 [$P < 0.001$] and 16.1 ± 8.2 vs. 8.6 ± 3.2 [$P < 0.001$], respectively) (Fig. 2B).

Effect of MAPK inhibitors on MRSA supernatant-induced MUC5AC protein production

To reveal MRSA-induced activation of cell signaling, we examined the MAPK pathway that concerned MUC5AC protein production. The cells were treated with or without MAPK inhibitors (ERK inhibitor, p38 MAPK inhibitor, or JNK inhibitor), and the protein level of MUC5AC was evaluated by ELISA. Compared with the stimulation group, the ERK inhibitor significantly reduced MRSA-induced protein production to the same protein level as in the control at concentration of 10μM (88.7 ± 22.9% greater than control vs. 3.5 ± 37.1% greater than control, $P < 0.01$) (Fig. 3). The effect of ERK inhibitor was observed at concentration of 2 μM, but not at 0.4 μM (141.9 ± 11.2% greater than control [stimulation group] vs. -1.2 ± 5.3% greater than control [$P < 0.01$] and 159.3 ± 4.0% greater than control [not significant difference], respectively). In contrast, the p38 MAPK inhibitor and JNK inhibitor did not reduce protein production at concentration of 10μM (Fig. 3).

Linezolid inhibited the phosphorylation of ERK in MRSA supernatant–activated NCI-H292 cells

To demonstrate the effect of linezolid on the MAPK pathway, we examined the phosphorylation of MAPKs. As shown in Fig. 4A, the MRSA supernatant increased the phosphorylation of ERK1/2, which was inhibited by linezolid at a concentration of 5 μg/mL (Fig. 4A). In contrast, there was no significant change in the expression of p38 MAPK and JNK. The inhibitory effect of linezolid on the phosphorylation of ERK1/2 was also observed at a concentration of 20 μg/mL (Fig. 4B). Additionally, we also examined the activation of

MAPK pathway at 360 and 540 minutes in which MUC5AC mRNA overexpression was observed. At these times, only phosphorylation of ERK1/2 was observed (Fig. 5A). The anti–phospho-p38 MAPK and anti–phospho-JNK antibodies worked with positive control cell extracts (Fig. 5B). Then, an influence of MRSA supernatant on NF-kB pathway was examined. As shown in Fig. 6, phosphorylation of IkBa was not observed in the western blot analysis.

Discussion

In this study, the MRSA-induced MUC5AC overexpression was inhibited by linezolid. The inhibitory effect on pathogen-induced MUC5AC overexpression has been reported as one of the immunomodulatory effects of macrolides (9-12, 14). MUC5AC as well as MUC5B is gel-forming mucin that is strongly expressed in the lung (15). They are the major barrier that traps inhaled microbial organisms, particulates, and foreign irritants in airway epithelium. In particular, MUC5B may play important roles in an airway defense, because a loss of *Muc5b* gene reduced survival by causing bacterial infection in a murine model (16). In contrast, the survival of mice was unaffected by a loss of *Muc5ac* gene. However, MUC5AC overexpression was observed in the *Muc5b*$^{-/-}$ mice, and it caused abnormal breathing and hypoxemia by impaired mucociliary clearance (16). In patients with chronic respiratory diseases such as, diffuse panbronchiolitis and asthma, MUC5AC overexpression was also observed (17, 18). In these diseases, mucin overexpression would cause airway obstruction, atelectasis, reduction of oxygenation, and reduction of antibiotic permeability. The overexpression is induced by various pathogens, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Haemophilus influenzae*, *Fusobacterium nucleatum*, *Chlamydophila pneumoniae*, *and Legionella pneumophila* (9-14, 20, 21). Thereby, it is very important to control pathogens-induced MUC5AC overexpression in patients with respiratory infectious diseases, and the inhibition of the overexpression was considered as the immunomodulatory effect of linezolid.

The immunomodulatory effect of linezolid was reported in several studies. Some studies have shown that linezolid reduces the level of lipopolysaccharide-induced production of inflammatory cytokines in whole blood (5-7). Additionally, in the previous studies with methicillin-sensitive *S. aureus* and MRSA, linezolid has been shown to have an inhibitory effect on the pathogens-induced production of inflammatory cytokines (2-4). However, in

these studies, bacteria were co-cultured with linezolid, and linezolid also reduced the levels of toxin production (2-4). As a result, it was concluded that the inhibitory effect of linezolid on the production of cytokines is associated with a reduction in toxin production. In this study, we used a supernatant of MRSA culture without linezolid to avoid the direct effect of linezolid on MRSA, including the effect on toxin production. Accordingly, our results showed the direct immunomodulatory effect of linezolid on airway epithelial cells.

Although *S. aureus* is one of the important pathogens causing respiratory tract infection or pneumonia, there have been few reports regarding the influence of *S. aureus* on MUC5AC production; one study used an *S. aureus* supernatant (20), and another used peptidoglycan from *S. aureus* (22). In this study, we revealed the mechanism of the overexpression. The mechanisms of MUC5AC overexpression in airway epithelial cell lines due to activation of MAPK pathway such as p38 MAPK and ERK1/2 has been reported at various stimulates (27), and the previous study using peptidoglycan from *S. aureus* reported that peptidoglycan-induced MUC5AC expression was activated the ERK1/2 pathway (22). This finding was similar to our results; in this study, only the ERK1/2 inhibitor reduced MUC5AC overexpression at the protein level. In western blot analysis, ERK1/2 was phosphorylated and p38 MAPK and JNK were not phosphorylated. Thus, activation of the ERK1/2 pathway seems to be specific to *S. aureus*-induced MUC5AC overexpression. Furthermore, we showed that the mechanism of the effect of linezolid: linezolid inhibited the phosphorylation of ERK1/2 in western blot analysis. The upper stream factors of ERK1/2, which was affected by MRSA and linezolid, was not investigated in this study, but the findings about the inhibitory effect of linezolid in MAPK pathway could suggest the direct effect of linezolid on airway epithelial cells.

There are some limitations in this study. We used only 1 MRSA strain that is a clinical isolate obtained from our hospital, and there is some possibility that the MUC5AC

overexpression was strain specific. We did not compare the effect of linezolid with the other anti-MRSA agents, and it was not recognized whether the effect is a specific superior characteristics. However, we think that our results showed a possibility that the immunomodulatory effect of linezolid helps a treatment in the critically ill patients. In this study, linezolid showed the inhibition effect at a trough concentration in the ELF of patients with VAP (25). In patients with VAP, MRSA is the most common pathogen, and the mortality rate is as high as 32.2% (28). Additionally, mucin overexpression was also observed in the patients, and the overexpression contributes to impending mucociliary clearance and favor the colonization of *Pseudomonas aeruginosa* that is also the common pathogen in VAP (19). Consequently, the control of mucin overexpression seemed to be important in such patients, and linezolid might have a potential to inhibit the overexpression.

In conclusion, our study showed that the MRSA supernatant-induced MUC5AC expression via activation of the ERK1/2 pathway and that linezolid inhibits MUC5AC overexpression. The inhibition effect was considered as a direct immunomodulatory effect on airway epithelial cells, and the effect has a possibility to help a treatment in patients with VAP caused by MRSA.

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Figure legends

Fig. 1. Influence of the MRSA supernatant on MUC5AC protein production and gene expression.

The NCI-H292 cells were stimulated with 1/80-, 1/40-, and 1/20-fold diluted solution of the MRSA supernatant. In the control, cells were stimulated with the same amount of LB broth as the supernatant. (A) After 24 hours of stimulation, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Of the 3 concentrations, only the 1/40-fold dilute solution significantly increased the protein level of MUC5AC. (B) Cells were stimulated with the 1/40-fold dilute solution of the MRSA supernatant for 3, 6, and 9 hours. The mRNA level of MUC5AC was assayed by RT-PCR. The MRSA supernatant significantly increased MUC5AC mRNA expression at 6 and 9 hours. Results are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$.

ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain reaction

Fig. 2. Effect of linezolid on MRSA supernatant-induced MUC5AC protein and gene overexpression.

The NCI-H292 cells were treated with linezolid (5 μg/mL and 20 μg/mL). In the control, the cells were treated with culture medium only. (A) After 24 hours of treatment, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Linezolid significantly reduced the MRSA supernatant-induced protein production at both concentrations. (B) The cells were treated with linezolid for 6 hours, and the mRNA level of MUC5AC was assayed by RT-PCR. Linezolid inhibited MRSA supernatant-induced MUC5AC mRNA expression at both concentrations. Results are

expressed as the mean ± SD. *$P < 0.001$ compared with the stimulation group.
ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant *Staphylococcus aureus*; RT-PCR, reverse-transcription polymerase chain reaction

Fig. 3. Effect of MAPK inhibitors on MUC5AC protein overexpression.

The cells were treated with an ERK inhibitor, p38 MAPK inhibitor, or JNK inhibitor at concentration of 10μM. After 24 hours, the protein levels were evaluated by ELISA. Only the ERK inhibitor significantly reduced MRSA-induced MUC5AC protein production compared with the stimulation group. Results are expressed as the mean ± SD. *$P < 0.01$ compared with the stimulation group.
ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

Fig. 4. Effect of linezolid on the MAPK pathway.

The NCI-H292 cells were treated with or without linezolid for 0, 30, 60, or 90 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant increased only phosphorylation of ERK1/2. The phosphorylation of ERK1/2 was inhibited by linezolid at a concentration of 5 μg/mL. There were no significant changes in other MAPKs, such as p38 MAPK and JNK. (B) The inhibitory effect of linezolid on the phosphorylation of ERK1/2 was observed at concentrations of 5 μg/mL and 20 μg/mL at 60 and 90 minutes. ERK, extracellular signal–regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

Fig. 5. Influence of MRSA supernatant on the MAPK pathway.

The NCI-H292 cells were stimulated with MRSA supernatant for 0, 180, 360, or 540 minutes, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant increased only phosphorylation of ERK1/2 at 180, 360, and 540 minutes. In contrast, there were no significant changes in p38 MAPK and JNK. (B) The anti–phospho-p38 MAPK and anti–phospho-JNK antibodies worked with positive control cell extracts.

ERK, extracellular signal–regulated protein kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRSA, methicillin-resistant *Staphylococcus aureus*.

Fig. 6. Influence of MRSA supernatant on the NF-kB.

The NCI-H292 cells were stimulated with MRSA supernatant, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant did not activate the NF-kB pathway at 30, 60, and 90 minutes. (B) The MRSA supernatant did not activate the NF-kB pathway at 30, 60, and 90 minutes. (C) The anti–phospho-IkBa MAPK antibodiy worked with positive control cell extracts.

NF-kB, NF-kappa-B; IkBa, I-kappa-B alpha; MRSA, methicillin-resistant *Staphylococcus aureus*.

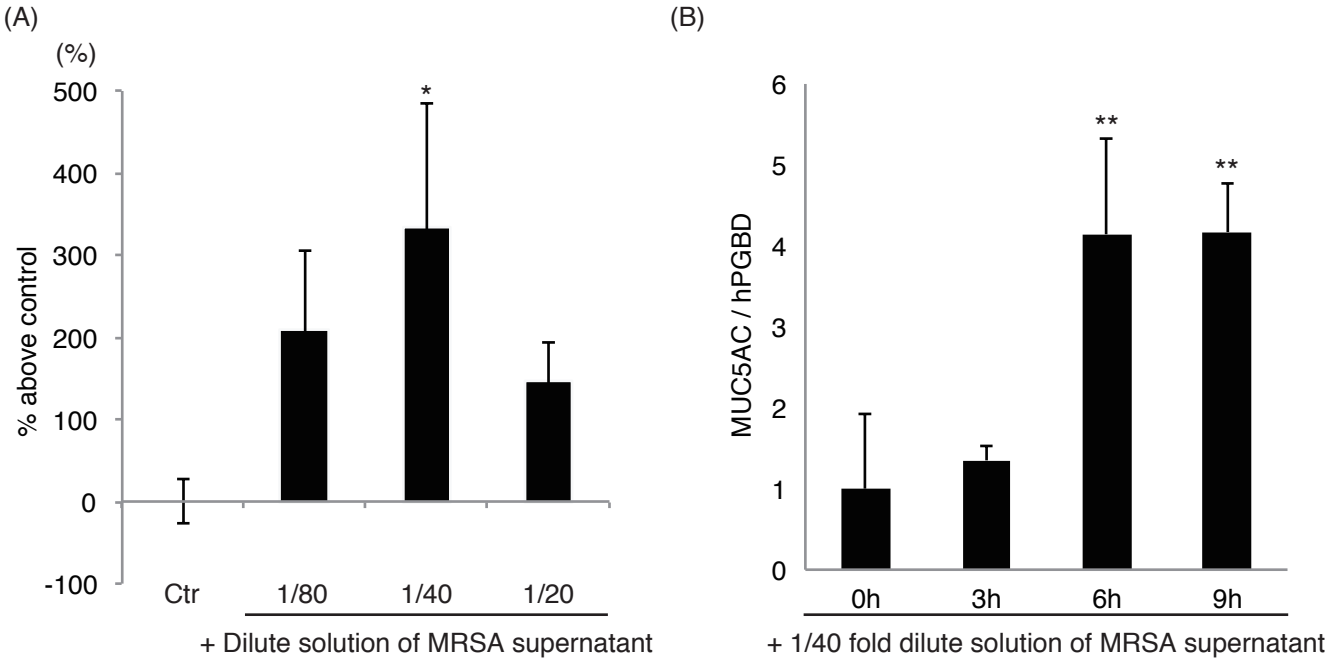


Fig. 1. Influence of the MRSA supernatant on MUC5AC protein production and gene expression.
The NCI-H292 cells were stimulated with 1/80-, 1/40-, and 1/20-fold diluted solution of the MRSA supernatant. In the control, cells were stimulated with the same amount of LB broth as the supernatant. (A) After 24 hours of stimulation, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Of the 3 concentrations, only the 1/40-fold dilute solution significantly increased the protein level of MUC5AC. (B) Cells were stimulated with the 1/40-fold dilute solution of the MRSA supernatant for 3, 6, and 9 hours. The mRNA level of MUC5AC was assayed by RT-PCR. The MRSA supernatant significantly increased MUC5AC mRNA expression at 6 and 9 hours. Results are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$. ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant Staphylococcus aureus; RT-PCR, reverse-transcription polymerase chain reaction

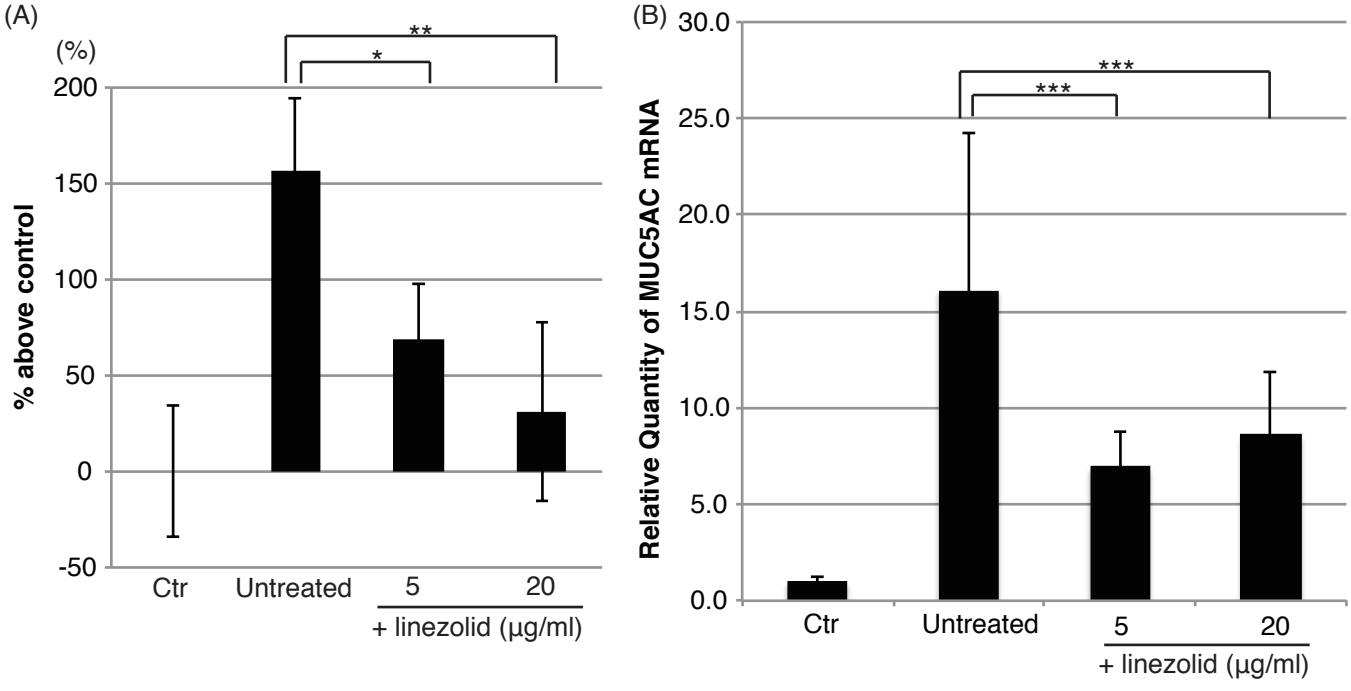
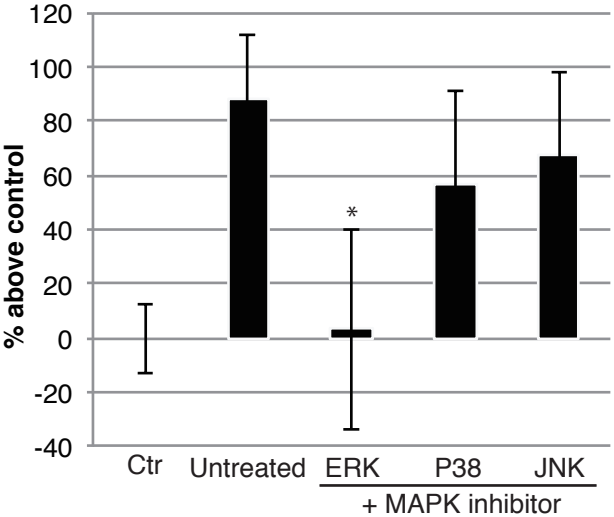


Fig. 2. Effect of linezolid on MRSA supernatant-induced MUC5AC protein and gene overexpression.

The NCI-H292 cells were treated with linezolid (5 μg/mL and 20 μg/mL). In the control, the cells were treated with culture medium only. (A) After 24 hours of treatment, the protein levels were measured by ELISA and provided in terms of percentage greater than control (n = 3). Linezolid significantly reduced the MRSA supernatant-induced protein production at both concentrations. (B) The cells were treated with linezolid for 6 hours, and the mRNA level of MUC5AC was assayed by RT-PCR. Linezolid inhibited MRSA supernatant-induced MUC5AC mRNA expression at both concentrations. Results are expressed as the mean ± SD. *$P < 0.001$ compared with the stimulation group.

ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; MRSA, methicillin-resistant Staphylococcus aureus; RT-PCR, reverse-transcription polymerase chain reaction



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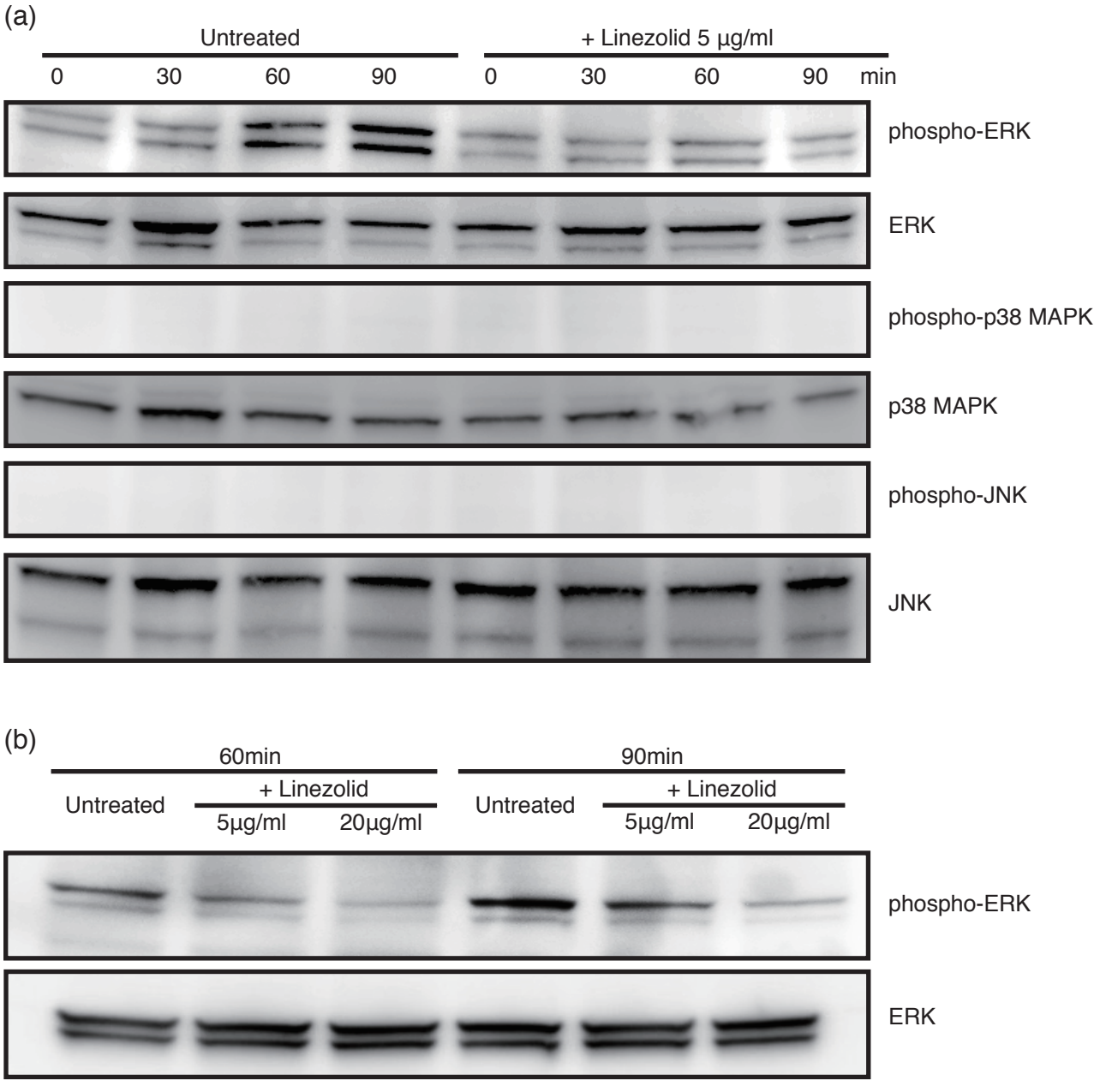

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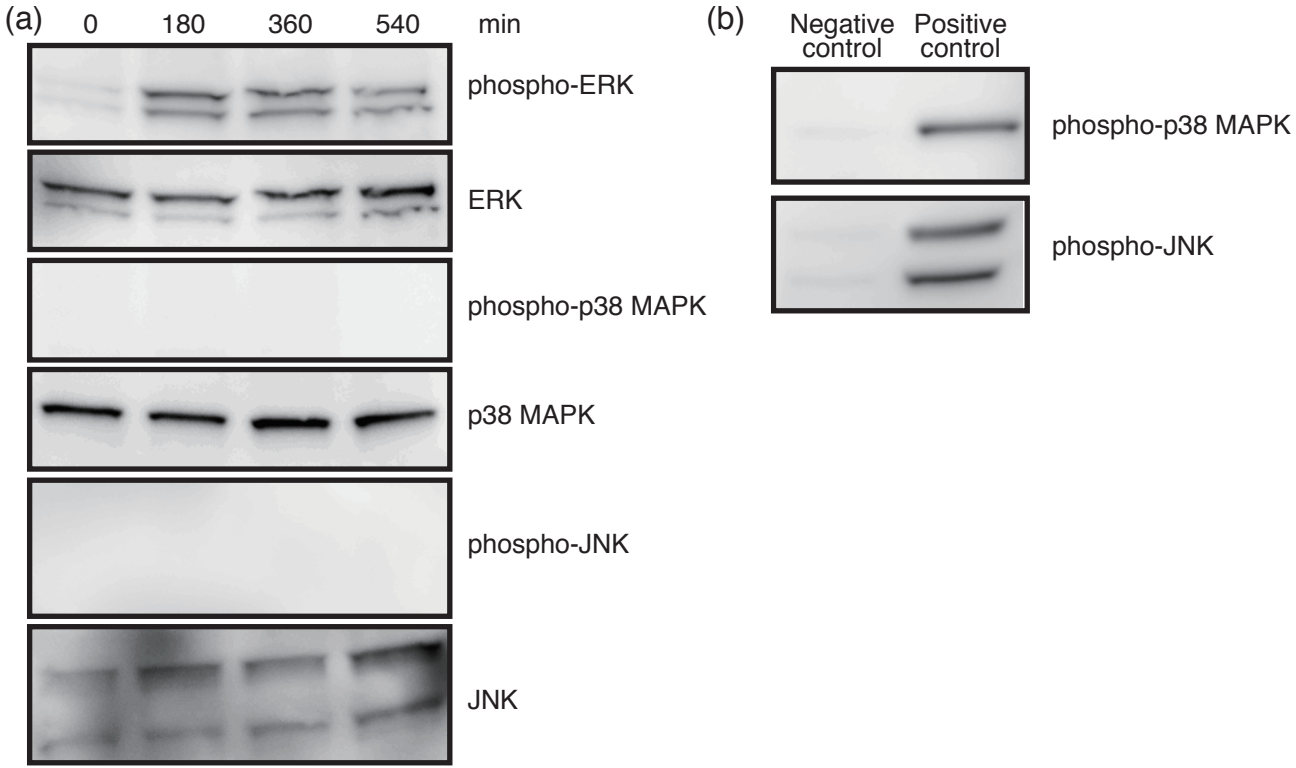

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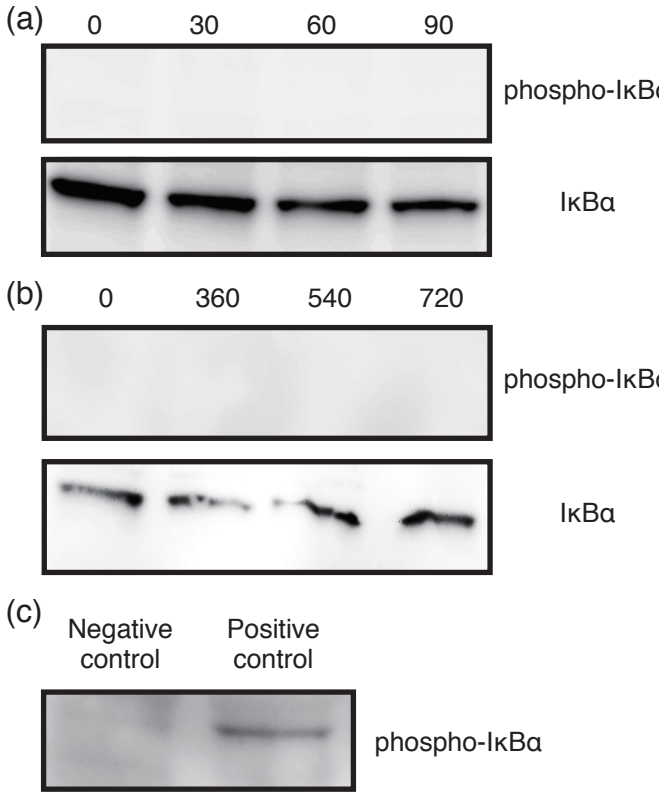

Fig. 6. Influence of MRSA supernatant on NF-kB

The NCI-H292 cells were stimulated with MRSA supernatant, and the cells were harvested after treatment and evaluated by western blotting. (A) The MRSA supernatant did not activate the NF-kB pathway at 30, 60, and 90 minutes. (B) The MRSA supernatant did not activate the NF-kB pathway at 30, 60, and 90 minutes. (C) The anti–phospho-IkBa MAPK antibodiy worked with positive control cell extracts.

NF-kB, NF-kappa-B; IkBa, I-kappa-B alpha; MRSA, methicillin-resistant Staphylococcus aureus.