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Original Article

The Effect of Doxycycline on PMA-Induced MUC5B Expression via MMP-9 and p38 in NCI-H292 Cells

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Objectives. Doxycycline is commonly used in medicine for its bacteriostatic antimicrobial properties. Recent studies have reported that doxycycline also has anti-inflammatory effects. Matrix metalloproteinase (MMP)-9 has been found to be involved in the physiological and pathological process of inflammatory airway disease. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, is known to stimulate the expression of MMP and mucin genes in the airway and intestinal epithelial cells. Therefore, the effects and signal pathways of doxycycline on PMA-induced MUC5B expression dependent MMP-9 in human airway epithelial cells were investigated.

Methods. In human NCI-H292 airway epithelial cells, MUC5B and MMP-9 mRNA expression, MUC5B protein expression, and MMP-9 protein activity after the treatment with PMA, MMP-9 or doxycycline were determined by reverse transcriptase-polymerase chain reaction, enzyme immunoassay, gelatin zymography, and Western blot analysis.

Results. PMA increased MMP-9 and MUC5B expression. MMP-9 increased MUC5B expression. Doxycycline inhibited PMA-induced MUC5B expression, and PMA-induced MMP-9 mRNA expression and protein activity. Doxycycline inhibited phosphorylation of p38 induced by PMA and MMP-9.

Conclusion. The results of this study suggest that doxycycline inhibited PMA-induced MUC5B mRNA expression and protein production through the MMP-9 and p38 pathways in human NCI-H292 airway epithelial cells.

Key Words. *Doxycycline, Inflammation, Phorbol myristate acetate, Matrix metalloproteinase-9, p38, Mucins, MUC5B, Epithelial cells, NCI-H292 cell*

INTRODUCTION

Respiratory mucus secretion is essential for protecting the lungs and airway tracts from external environment, chemicals, and microorganisms. However, mucus secretion is abnormally augmented in disease states, such as chronic bronchitis, asthma, cystic fibrosis, and chronic rhinosinusitis, which lead to an increase in the morbidity and mortality of the affected patients (1, 2). The predominant mucins present in inflammatory airway disease are MUC4, MUC5AC, and MUC5B, which are regulated by many pathophysiological mediators and hormones (3).

Among the pathophysiological mediators of mucin secretion, matrix metalloproteinases (MMPs) play a critical role in the maintenance and turnover of extracellular matrix macromolecules. MMPs are associated with inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis. Especially, in inflammatory airway disease, the predominant form of MMPs is MMP-9 (4-6). However, the precise role and function of MMP-9 with regard to mucin secretion in inflammatory airway disease remains unknown.

Phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, has been used as an inflammatory stimulant that can modulate a variety of cellular events including gene transcription, cell growth, and differentiation (7). PMA also

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stimulates MMP-9 expression in various cancer cells, and MUC2 and MUC5B expression in airway and intestinal epithelial cells (7-10).

Doxycycline, a long-acting semisynthetic tetracycline, is commonly used in medical fields because of its safety and efficacy as a bacteriostatic antimicrobial agent. In addition to these effects, recent studies have demonstrated that doxycycline has both intracellular and extracellular anti-inflammatory effects (11, 12). However, the effects of doxycycline on mucin secretion in inflammatory airway disease have not been clearly defined.

Therefore, the goal of this study was to determine whether doxycycline might play an important role in mucin secretion of the inflammatory airway epithelial cells. In this study, the effects and signal pathways of doxycycline on PMA-induced MUC5B expression dependent MMP-9 in human airway epithelial cells were investigated.

MATERIALS AND METHODS

Cell culture and treatment

The mucin-producing human NCI-H292 airway epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA). The cells were grown at 37°C in 5% CO_2 fully humidified air, and were subcultured twice weekly. The cells were seeded in a 6-well plate at 1×10^5 cells/well. When the cultured cells were in a confluent state, the cultured cells were incubated in RPMI 1640 medium containing 0.5% FBS for 24 hours. The cultured cells were then rinsed with serum-free RPMI 1640 medium and exposed to the indicated concentrations of PMA (Sigma, St. Louis, MO, USA), MMP-9 (R&D Systems Inc., Minneapolis, MN, USA), and doxycycline (Sigma). In order to investigate the signaling pathway of PMA-induced MUC5B expression, SB2035 (BOMOL Research Laboratories, Plymouth Meeting, PA, USA), as specific inhibitors, was used to pretreat the human NCI-H292 airway epithelial cells for 1 hour before exposure to the indicated concentrations of PMA. In the case of the controls, the cultured cells were incubated with medium alone for the same amount of time.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of MUC5B and MMP-9mRNA expression

The total RNAs from PMA treated the cultured cells were prepared using the available reagent (TRIzol; Invitrogen) according to the manufacturer's instructions. Fifteen micrograms of the RNA were reverse-transcribed into cDNA at 37°C for 70 minutes in 60 μL volume of reaction mixture that contained 150 U of reverse-transcriptase (Superscript-II; Invitrogen), 10 mM each of dATP, dTTP, dCTP, and dGTP, and 5 μL of 50 μM Oligo-dT primer/mL (Amersham International PLC, Little Chalfont, Buckinghamshire, UK). The reactions were stopped by heat inactivation at 85°C for 10 minutes. Two microliters of each cDNA sample, from the reverse transcription, were amplified by PCR (PTC-200, MJ Research Inc., Watertown, MA, USA) in a volume of 50 μL that contained 0.5 U of Taq DNA polymerase, 2 μL of 50 mM $MgCl_2$, 1 μL each 0f 10 mM dATP, dTTP, dCTP, and dGTP, and 1 μL of the 10 μM primers. The PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on each individual sample as a positive internal control. The primer sequences used in the PCR were 5′-CAC ATC CAC CCT TCC AAC-3′ (sense) and 5′-GGC TCA TTG TCG TCT CTG-3′ (antisense) for MUC5B (245 bp), and 5′-CCT CCA AGG AGT AAG ACC CC-3′ (sense) and 5′-AGG GGT CTA CAT GGC AAC TG-3′ (antisense) for GAPDH (145 bp). After a hot start, the amplification profile was 30 cycles for 1 minute of denaturation at 95°C, 30 seconds of annealing at 60°C, and 1 minute of extension at 72°C in the presence of 2.5 mg $MgCl_2$ for MUC5B and MMP-9. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by UV fluorescence. Semiquantitative analysis of the RT-PCR product was performed on the scanned gel images, and the intensity of the PCR product was measured using commercially available imaging software (Scion Software; Scion Co., Frederick, MD, USA). The relative intensity of the individual bands on the gel image was determined as the ratio of the intensities of MUC5B and MMP-9 to the intensity of GAPDH.

Enzyme-linked immunosorbent assay (ELISA) analysis of MUC5B protein

MUC5B protein was determined by ELISA. Samples of the supernatant or cell lysates from the cultured cells were prepared in phosphate-buffered saline (PBS) at several dilutions, and each sample was incubated at 40°C in a 96-well plate until dry. The plates were then washed three times with PBS, blocked with 2% bovine serum albumin for 1 hour at room temperature, washed again three times with PBS, and incubated with primary antibody of MUC5B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted with PBS containing 0.05% Tween 20 for 1 hour. The wells were then washed three times with PBS and treated with horseradish peroxidase-conjugated secondary antibody of MUC5B (Santa Cruz Biotechnology). After 4 hours, the plates were washed three times with PBS. Color was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution and stopped with 2N-H_2SO_4. The absorbance was read at 450 nm.

Gelatin zymography assay analysis of MMP-9 protein activity

MMP-9 protein activity was measured by gelatin zymography. In brief, the cells culture supernatants containing 10 μg of protein were mixed with 2×sodium-dodecyl sulfate loading buffer before being loaded onto 10% polyacrylamide gels (wt/vol)

containing 0.1% (wt/vol) gelatin as a substrate (Biotech, Piscataway, NJ, USA). The protein was subjected to electrophoresis at 80 volt for 120 minutes and the gel was then washed twice with zymogram renaturing solution (Biotech) for 45 minutes at room temperature. The gel was preincubated in zymogram developing solution (Biotech) for 30 minutes at 37°C and subsequently incubated in zymogram developing solution for 24 hours at 37°C. After incubation, the gel was stained by 0.5% Coomassie Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) in 30% methanol and 5% acetic acid solution under continuous shaking for 2 hours, then de-stained in 30% methanol and 5% acetic acid solution for 30 minutes, and then rinsed twice with destaining solution to visualize the digested bands in the gelatin matrix. The gels were photographed and the averages of the band intensity were measured using commercially available imaging software (Scion Software).

Western blot analysis of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 phosphorylation

The cultured cells were seeded in a 6-well plate and treated with PMA and/or MMP-9, and doxycycline for the indicated times. The cells were then washed with cold PBS, exposed to trypsin, and formed into pellets at 700 g at 4°C. The pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The preparation was then clarified by centrifugation, and the supernatant was saved as a whole-cell lysate. The proteins (50 μg) were separated using 10% reducing SDS-polyacrylamide gel electrophoresis and electroblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in 25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20, and then incubated with the indicated primary antibody of ERK1/2 (Santa Cruz Biotechnology) or p38 (Santa Cruz Biotechnology) for 4 hours. Subsequently, the membrane was washed and incubated for 1 hour with secondary antibody of ERK1/2 (Santa Cruz Biotechnology) or p38 (Santa Cruz Biotechnology) conjugated to HRP, rewashed, and developed using an enhanced chemiluminescence reagent kit (PerkinElmer Life Sciences, Boston, MA, USA) and exposed to X-ray film for 10 seconds.

Cell transfection with small interfering RNA (siRNA) for p38 mitogen-activated protein kinase (MAPK)

Predesigned siRNA targeting p38 MAPK and negative control siRNA for p38 MAPK were purchased from Invitrogen (Carlsbad, CA, USA). The sequences of p38 MAPK siRNA were as follows; sense: AUG AAU GAU GGA CUG AAA UGG UCU G and antisense: CAG ACC AUU UCA GUC CAU CAU UCA U. The transfection rate of p38 MAPK siRNA was verified to be over 90% in the human NCI-H292 airway epithelial cells. Transfection was performed according to the manufacture's protocol (Invitrogen). In brief, the human NCI-H292 airway epithelial cells were seeded in a 6-well plate at 1×10^5 cells/well and incubated overnight in RPMI 1640 medium without antibiotics. When the cells were 80-90% confluent, the following day, and the cells were washed with PBS, and then OPTI-MEN I Reduced Serum Medium (Invitrogen) was added to the cells. Then, p38 MAPK siRNA and a nucleic acid transferring agent, Lipofectamine 2000 (Invitrogen) was incubated together in OPTI-MEM I Reduced Serum Medium for 20 minutes at room temperature to form a p38 MAPK siRNA-Lipofectamine complex. The p38 MAPK siRNA-Lipofectamine complex-containing medium was added to each well containing the cells to a final p38 MAPK siRNA concentration of 20 nM; the cells were then incubated for 24 hours at 37°C in a CO_2 incubator. The p38 MAPK siRNA-Lipofectamine complex-containing medium was replaced with RPMI 1640 medium after 4 hours without loss of transfection activity. After 24 hours of transfection with p38 MAPK siRNA, the cells were exposed to the indicated concentrations of PMA and then harvested for RT-PCR analysis of MUC5B mRNA expression. The same procedure was performed with a negative control siRNA (Invitrogen).

Statistical analysis

Statistical analysis was performed using SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). The mean for each of the obtained quantitative values was calculated. Comparisons were made using the Student's *t*-test. For all tests, a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of PMA on MUC5B and MMP-9 mRNA expression

The effects of PMA on MUC5B and MMP-9 mRNA expression in a dose-dependent manner were investigated. The MUC5B mRNA expression was significantly increased at all doses of PMA and peaked to 10 nM of PMA for 8 hours, and the MMP-9 mRNA expression was significantly increased about 17 fold at 5, 10, 25, and 50 nM for 8 hours ($P<0.05$) (Fig. 1).

Effect of MMP-9 on MUC5B mRNA expression

The effect of MMP-9 on MUC5B mRNA expression in a dose-dependent manner was investigated. MUC5B mRNA expression was significantly increased in a dose-dependent manner ($P<0.05$) (Fig. 2).

Phosphorylation of p38 in PMA-induced MUC5B expression

SB203580 as a p38 MAPK inhibitor inhibited the PMA-induced MUC5B expression ($P<0.05$) (Fig. 3A). In addition, the knockdown of p38 MAPK by p38 MAPK siRNA significantly blocked PMA-induced MUC5B mRNA expression ($P<0.05$) (Fig. 3B).

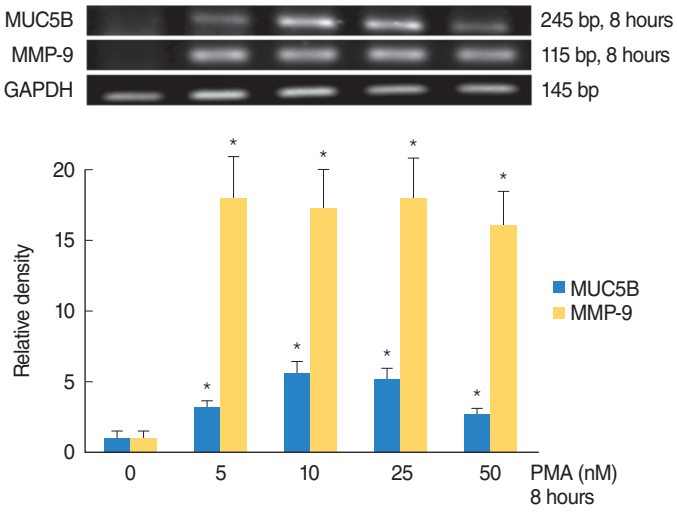

Fig. 1. The effect of phorbol 12-myristate 13-acetate (PMA) on MUC5B and matrix metalloproteinase (MMP)-9 mRNA expression. Human NCI-H292 airway epithelial cells were stimulated with PMA. MUC5B and MMP-9 mRNA levels were analyzed by RT-PCR. MUC5B mRNA express ion was significantly increased at all doses of PMA and peaked at 10 nM of PMA. MMP-9 mRNA expression was significantly increased about 17 fold at 5, 10, 25, and 50 nM of PMA. *P<0.05 compared with zero value.

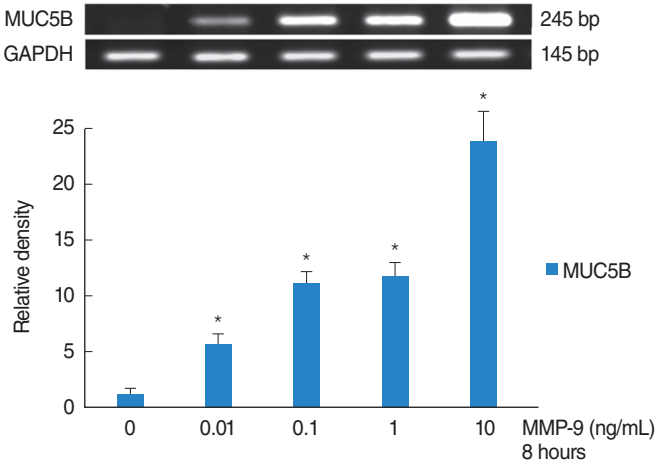

Fig. 2. The effect of matrix metalloproteinase (MMP)-9 on MUC5B mRNA expression. Human NCI-H292 airway epithelial cells were stimulated with MMP-9. MUC5B mRNA levels were analyzed by RT-PCR. MUC5B mRNA expression was significantly increased in a dose-dependent manner. *P<0.05 compared with zero value.

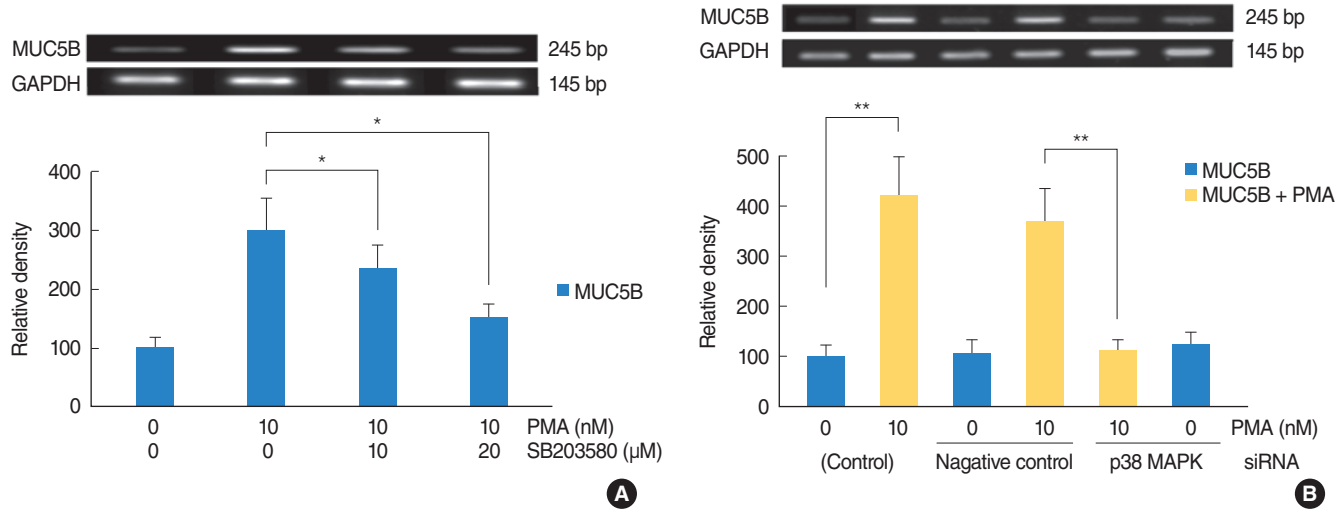

Fig. 3. The effect of SB203580 and p38 MAPK siRNA on the phosphorylation of p38 MAPK in phorbol 12-myristate 13-acetate (PMA)-induced MUC5B mRNA expression. Human NCI-H292 airway epithelial cells were stimulated with SB203580 before exposure to PMA, and also were transfected with predesigned siRNA targeting p38 MAPK and a negative control siRNA for p38 MAPK before exposure to PMA. MUC5B mRNA expression was analyzed by RT-PCR. (A) SB203580 inhibited PMA-induced MUC5B expression. (B) The knockdown of p38 MAPK by p38 MAPK siRNA significantly blocked PMA-induced MUC5B mRNA expression. *P<0.05 compared with PMA alone. **P<0.05 compared with control.

Effect of doxycycline on PMA-induced MUC5B expression and PMA-induced MMP-9 mRNA expression and protein activity

Doxycycline significantly inhibited PMA-induced MUC5B expression in a dose-independent manner (P<0.05) (Fig. 4). Doxycycline significantly inhibited PMA-induced MMP-9 mRNA expression (P<0.05) (Fig. 5A). In addition, doxycycline significantly inhibited PMA-induced MMP-9 protein activity (P<0.05) (Fig. 5B).

Phosphorylation of ERK1/2 and p38 in the effect of doxycycline after treatment with PMA or MMP-9

Doxycycline inhibited PMA induced phosphorylation of p38 in a dose dependent manner; however, it did not change the phosphorylation of ERK1/2 significantly (P<0.05) (Fig. 6A). In addition, doxycyline significantly inhibited MMP-9 induced phosphorylation of p38 (P<0.05) (Fig. 6B).

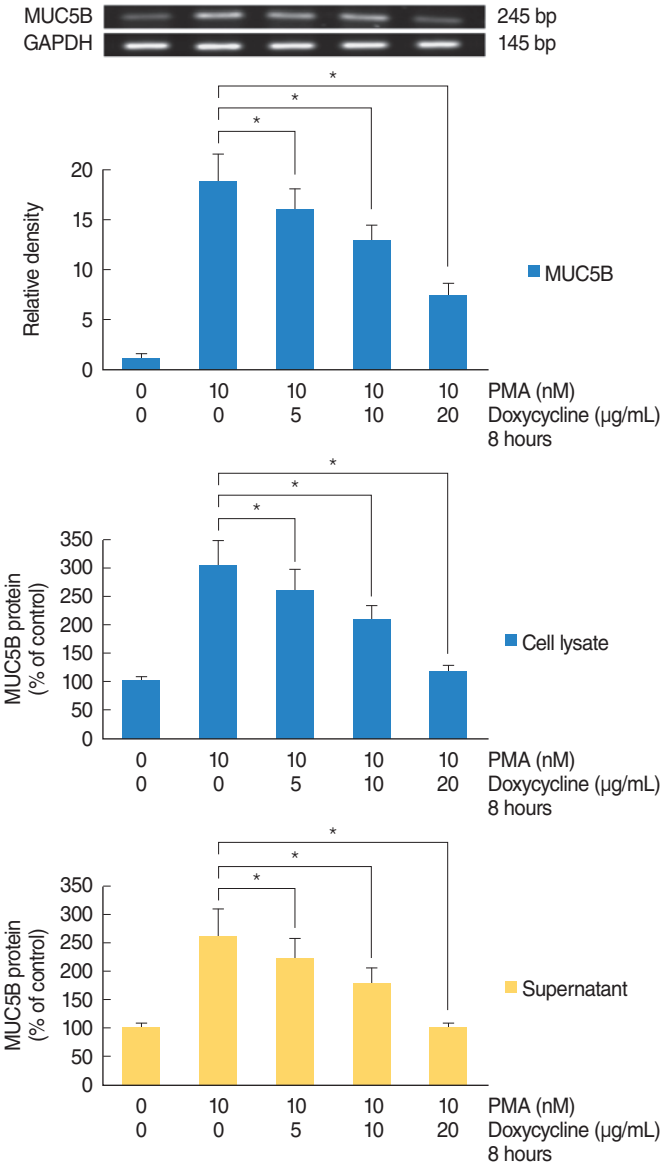

Fig. 4. The effect of doxycycline on phorbol 12-myristate 13-acetate (PMA)-induced MUC5B expression. Human NCI-H292 airway epithelial cells were stimulated with doxycycline after the treatment of PMA. MUC5B RNA levels were analyzed by RT-PCR, and MUC5B protein levels of cell lysates and supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). Doxycycline significantly inhibited PMA-induced MUC5B mRNA expression and protein production in a dose-dependent manner. *P<0.05 compared with PMA alone.

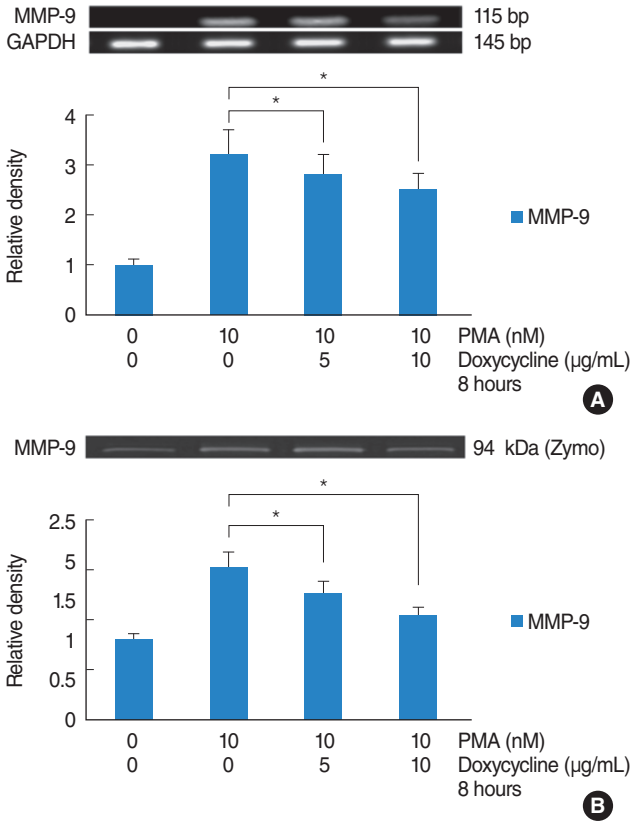

Fig. 5. The effect of doxycycline on phorbol 12-myristate 13-acetate (PMA)-induced matrix metalloproteinase (MMP)-9 mRNA expression and protein activity. Human NCI-H292 airway epithelial cells were stimulated with doxycycline after treatment with PMA. MMP-9 mRNA levels were analyzed by RT-PCR, and MMP-9 protein activity was measured by gelatin zymography. (A) Doxycycline significantly inhibited PMA-induced MMP-9 mRNA expression. (B) Doxycycline significantly inhibited PMA-induced MMP-9 protein activity. *P<0.05 compared with PMA alone.

DISCUSSION

MUC5B is located on chromosome 11p15.5, and induce the major gel-forming mucin that plays an important role in the protection and lubrication of the epithelial surface of the normal human airway (13). MUC5B also induce mucin secretion in chronic bronchitis and cystic fibrosis by a variety of inflammatory mediators such as bacterial lipopolysaccharide (LPS), interleukin-1β, tumor necrosis factor-α and leukotriene D_4 (3, 14-16).

PMA is a PKC activator, and is used as a model inflammatory mediator that can modulate gene transcription, cell growth, and differentiation of cells (7). PMA is also known to stimulate the expression of MUC2 and MUC5B in the airway and intestinal epithelial cells, and the expression of MMP-9 in the airway and intestinal epithelial cells. And the p38 MAPK pathways are generally activated by stress related conditions, in various airway diseases, and are also involved in the regulation of PMA-induced MUC5B expression (7-10). Therefore, this study focused on MUC5B expression and p38 MAPK pathways in the effects and signal pathways of doxycycline on PMA-induced mucin secretion.

MMP-9 plays an important role in the regulation of extracellular matrix macromolecules, and is associated with persistent mucin production in human airway cells, and the regulation of MUC2 expression in the goblet cell of the colon (17, 18).

The precise mechanisms, associated with the regulation of the mucin gene for PMA and MMP-9, are unknown, but several

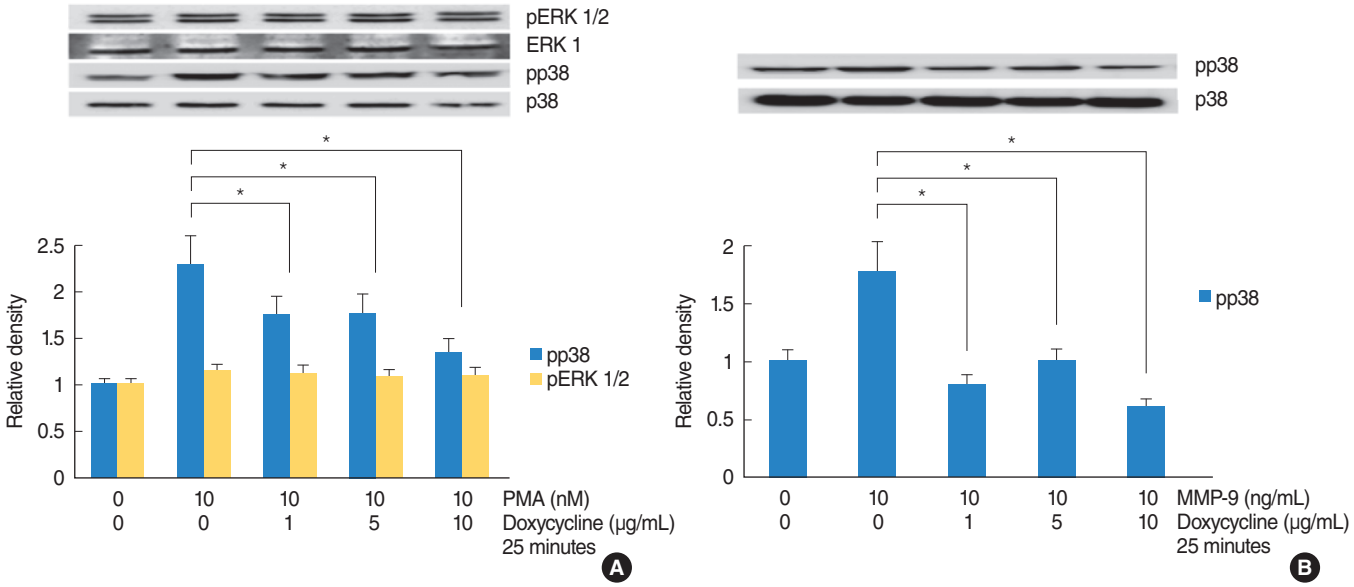

Fig. 6. The effects of doxycycline on the phosphorylation of ERK1/2 and p38 after treatment with phorbol 12-myristate 13-acetate (PMA) or matrix metalloproteinase (MMP)-9. Human NCI-H292 airway epithelial cells were stimulated with doxycycline after treatment with PMA or MMP-9. The phosphorylation of ERK1/2 and p38 were detected by Western blot analysis. (A) Doxycycline inhibited PMA induced phosphorylation of p38 in a dose dependent manner, but it did not change the phosphorylation of ERK1/2 significantly. (B) Doxycycline significantly inhibited MMP-9 induced phosphorylation of p38. *P<0.05 compared with PMA alone.

studies have reported that PMA induced MMPs expression, and then PMA-induced MMPs cause mucin gene expression through Sp1-dependent mechanisms (7, 19). In this study, PMA increased MUC5B and MMP-9 mRNA expression, and MMP-9 increased MUC5B mRNA expression in human NCI-H292 airway epithelial cells. These results suggest that MMP-9 may be an important mediator of MUC5B expression, and that PMA increases MUC5B expression through MMP-9.

Doxycycline, a member of the tetracycline family, has a bacteriostatic antimicrobial effect with inhibition of inflammation, angiogenesis and apoptosis (11, 12). In addition, doxycycline inhibits MMP activity, and leads to the prevention of extracellular matrix component breakdown in various cells such as human endothelial cells, skin keratinocytes, and corneal epithelial cells (11). Recently, several studies have shown that doxycycline inhibits LPS-induced MMP-9 expression and LPS-induced MUC5AC expression in airway epithelial cells (20), and inhibits transforming growth factor-β1-induced MMP-9 expression in human corneal epithelial cells (12), and inhibits acrolein-induced MMP-9 and MUC5AC expression in tracheal epithelium (21). However, the effects of doxycycline on mucin secretion in inflammatory airway disease have not been clearly defined. The results of this study demonstrated that doxycycline inhibited PMA-induced MUC5B expression, and inhibited PMA-induced MMP-9 mRNA expression and protein activity. In addition, doxycycline inhibited PMA and MMP-9 induced-phosphorylation of p38; SB203580 (p38 MAPK inhibitor) inhibited PMA-induced MUC5B mRNA expression. In addition, the knockdown of p38 MAPK by p38 MAPK siRNA significantly blocked PMA-induced MUC5B mRNA expression, and the knockdown of p38 MAPK by p38 MAPK siRNA significantly blocked PMA-induced MUC5B mRNA expression. While a study has reported that the high concentration of PMA (50 ng/mL) induced phosphorylation of ERK1/2 in human NCI-H292 airway epithelial cells (22), the results of this study suggest that doxycycline inhibits MUC5B expression through inhibitory action on MMP-9 expression, and p38 plays a role in the signal pathway of down-regulation of MUC5B by doxycycline in inflammatory airway disease. However, the effects of doxycycline on other mucins and the precise signal pathways for the regulation of the mucin gene by doxycycline in the airway epithelial cells require further confirmation.

In conclusion, the results of this study suggest that doxycycline inhibits PMA-induced MUC5B expression through the MMP-9 and p38 pathways in human NCI-H292 airway epithelial cells. These findings provide information that doxycycline may play a role in the control of mucus hypersecreation through MMP-9 and p38 pathways in human airway epithelial cells.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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