

## Full Paper

Glycyrrhizin Attenuates Mucus Production by Inhibition of *MUC5AC* mRNA Expression In Vivo and In VitroYuki Nishimoto<sup>1</sup>, Akinori Hisatsune<sup>1</sup>, Hiroshi Katsuki<sup>1</sup>, Takeshi Miyata<sup>2</sup>, Kazumi Yokomizo<sup>2</sup>, and Yoichiro Isohama<sup>1,\*</sup><sup>1</sup>Department of Chemico-Pharmacological Sciences, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan<sup>2</sup>Laboratory of Presymptomatic Medical Pharmacology, Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

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**Abstract.** We have previously indicated that glycyrrhizin (GL), a major component of licorice, has glucocorticoid-like anti-inflammatory effects in cultured airway epithelial cells and suggested its usefulness in the treatment of inflammatory respiratory diseases. On the other hand, mucus hypersecretion in the respiratory tract and goblet cell hyperplasia in the airway epithelium contribute to the morbidity and mortality associated with airway inflammatory diseases. This study, therefore, aimed to examine the effects of GL on airway mucus hyperproduction and define the mechanisms behind these effects. In an in vivo model, GL significantly attenuated goblet cell hyperplasia and *MUC5AC* mRNA expression in mice treated with lipopolysaccharide or interleukin-4. In addition, GL significantly attenuated *MUC5AC* protein and mRNA expression by tumor growth factor (TGF)- $\alpha$  in cultured NCI-H292 cells. GL also attenuated TGF- $\alpha$ -stimulated *MUC5AC* promoter activity in a luciferase reporter gene assay, but did not affect the stability of *MUC5AC* mRNA. Taken together, we concluded that GL has an inhibitory effect on mucus hyperproduction both in vivo and in vitro and that GL-mediated inhibition may be mediated through the inhibition of *MUC5AC* gene transcription.

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.09344FP>]**Keywords:** glycyrrhizin, mucin, goblet cell, airway inflammation, *MUC5AC*

## Introduction

Goblet cell hyperplasia and mucus hyperproduction in airway epithelium are hallmarks of chronic inflammatory lung diseases, including chronic obstructive pulmonary disease (COPD) and asthma (1, 2). While mucins contribute to natural host defense of the airway by acting as a physical barrier, overproduction of mucins in pathological conditions leads to exacerbation of these diseases by impairing mucociliary clearance, causing severe airway narrowing. Twenty-one mucin genes have been identified to date (3). Among these, mucin 5AC (*MUC5AC*) is a major mucin in the human respiratory tract and is closely linked to goblet cell hyperplasia.

*MUC5AC* mRNA levels are known to be upregulated by cytokines, lipopolysaccharide (LPS), neutrophil elastase, cigarette smoking, or reactive oxygen species via various signaling pathways (4 – 11). However, there have been few reports describing pharmacological regulation that can decrease stimulant-induced *MUC5AC* gene expression in the airway.

Glycyrrhizin (GL), a triterpene glycoside from licorice root (*Glycyrrhiza glabra*), has been used clinically for more than 20 years in patients with chronic hepatitis in Japan (12). Several lines of evidence have suggested the usefulness of GL in the treatment of bronchitis and asthma. For example, it has been shown that GL inhibits nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity and interleukin (IL)-8 expression in cultured lung epithelial cells (13, 14). It has also been shown that orally administered GL inhibited the asthmatic responses in ovalbumin (OVA)-sensitized mice and reduced levels of the inflammatory cytokines

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IL-4 and IL-5 in bronchoalveolar lavage (BAL) fluid (15). In addition, intravenously administered GL inhibited LPS induced severe acute respiratory syndrome in mice (16). However, the effect of GL on mucus hyperproduction and goblet cell hyperplasia has not been systematically studied.

The first objective of this study was to examine the *in vivo* effects of GL on mucus hyperproduction and goblet cell hyperplasia in mice treated with LPS or IL-4. We demonstrated that GL attenuates mucus production in mice treated with either agent. We also examined the *in vitro* effect of GL on TGF- $\alpha$ -induced MUC5AC expression in human bronchial epithelial cells to determine the mechanism of the inhibitory effect of GL on mucus production.

## Materials and Methods

### *Reagents and animals*

GL was obtained from Fluka Chemie GmbH (>95% purity; Steinheim, Germany). Dexamethasone (DEX) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-4 and TGF- $\alpha$  were from R&D Systems Inc. (Minneapolis, MN, USA). Male ICR mice aged 7 weeks were obtained from the Kyudo Experimental Animal Center (Kumamoto). Animal experiments were performed according to the animal care guidelines of Kumamoto University.

### *Goblet cell hyperplasia models and application of drugs*

Two types of airway mucus hyperproducing mouse models were used in this study. One of them was prepared by intratracheal treatment with LPS. Mice were anesthetized with pentobarbital (45 mg/kg, *i.p.*), and then LPS (100  $\mu$ g) in sterilized saline (50  $\mu$ L) was intratracheally administered (17, 18). Mice were scarified 7 days later and the tracheae and lungs were isolated and subjected to further experiments. Saline, GL (15, 45, or 135 mg/kg) or DEX (1 mg/kg) was subcutaneously administered 30 min before the LPS treatment (day 0) and administered daily on days 1 to 6. The second model involved the intratracheal treatment of anesthetized mice with IL-4 (250 ng) dissolved in 50  $\mu$ L. Saline (19), GL, or DEX was administered as a single dose 30 min before the IL-4 instillation, and lung and tracheae were sampled 24 h after IL-4 treatment.

### *Histological examination*

Tracheae were fixed in 10% neutral formalin and embedded in paraffin, before 4- $\mu$ m sectioning and staining with the Alcian blue-periodic acid-Schiff (AB-PAS) stain for measuring mucus production. Quantitative

analysis was performed as previously described (20) with minor modifications. In brief, the stained sections were observed under a light microscope. The cells stained with AB-PAS were counted as goblet cells. The number of goblet cells in a 3-mm length of three tracheal sections was counted, and the average of the three was considered as the goblet cell number of each mouse. MUC5AC, a major component of mucus glycoprotein, was also visualized by immunohistochemical staining. Mouse anti-MUC5AC antibody (1:100, clone 45M1; Neomarker, Fremont, CA, USA) and biotinylated anti-mouse IgG were used as primary and secondary antibodies, respectively. Positively stained cells were regarded as goblet cells.

### *BAL and cell counting*

At designated time points, BAL was carried out after euthanizing the mice and then placing a 20-gauge catheter into the trachea through which 1 mL of saline was flushed back and forth three times. BAL leukocytes were collected by centrifugation (450  $\times$  g, 5 min) and counted with a hemocytometer.

### *Cell culture*

NCI-H292 human lung mucoepidermoid carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI1640 medium (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) (Sigma) and antibiotics (100 U/mL penicillin and 100  $\mu$ g/mL streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. A549 human lung carcinoma cells were obtained from Riken Bioresource Center (Ibaraki) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Nissui) with 10% FBS and antibiotics.

### *MUC5AC protein expression*

To determine the expression of MUC5AC protein, NCI-H292 cells were cultured in 35-mm dishes. After 24 h of serum deprivation, cells were treated with TGF- $\alpha$  (50 ng/mL) combined with vehicle, GL or DEX for 24 h. Incubation media and cell lysates were then collected separately. Incubation media were centrifuged for 5 min at 800  $\times$  g and the supernatants were used. Cells were lysed in PBS containing 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and then centrifuged for 10 min at 10,000  $\times$  g. The supernatant was used for further studies.

The amount of MUC5AC protein in the media and cell lysates was determined by slot blotting. Medium (200  $\mu$ L) or cell lysates (100  $\mu$ g protein) were blotted on polyvinylidene fluoride (PVDF) membranes using Bio dot SF (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The membranes were blocked with 2% bovine serum albumin and 0.05% Tween 20 in PBS for 1 h at room temperature and incubated with anti-MUC5AC antibody overnight at 4°C. The membrane was washed with 0.05% Tween in PBS and incubated with peroxidase conjugated anti-mouse IgG for 1 h. After three additional washes, the signal was developed with the ECL Advance™ Western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA) on the lumino-imaging analyzer (LAS-3000 mini; Fuji, Tokyo). Band intensities were analyzed with Image Gauge software (Fuji).

#### *MUC5AC mRNA expression*

NCI-H292 cells were treated with TGF- $\alpha$  (50 ng/mL) combined with vehicle, GL, or DEX for 24 h, after which total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). Random-primed cDNA was prepared from 0.5  $\mu$ g total RNA using the Prime Script Reverse Transcriptase (Takara Bio, Otsu). PCR amplification was carried out with SYBR® Premix Ex Taq™ (Takara Bio) and analyzed in 96-well optical reaction plates using the threshold cycle (Ct) method. A murine *MUC5AC* cDNA fragment was amplified with the 5'-cagccgagaggagggttgatct-3' and 5'-agtctctctccgctctctcaat-3' primer pair. A human *MUC5AC* cDNA fragment was amplified with the 5'-tccggcctcatctctcc-3' and 5'-acttggcactggtgctg-3' primer pair. A GAPDH cDNA fragment was amplified with the 5'-accatcttcaggagcgaga-3' and 5'-cagtctctgggtggcagtg-3' primer pair. The PCR protocol was as follows: 94°C for 1 min and 60°C for 30 s, followed by repeating this cycle 40 times. The results are given as a ratio of specific mRNA/GAPDH. The fluorescence was monitored at the end of each cycle to obtain a measure of the amount of PCR product formed. The thermocycler PCR data were analyzed with the software installed in the Chromo4 Real-Time detector (Bio-Rad Laboratories, Inc.), which determines the cycle number at which each sample reaches this threshold. The cycle number corresponding to the fluorescence threshold is inversely related to the logarithm of the initial template concentration.

#### *MUC5AC promoter assay*

The reporter plasmid containing the 5'-flanking region of the human *MUC5AC* gene (−4.0 kb to +68 bp) was a kind gift from Dr. Carol B. Basbaum (University of California, San Francisco, CA, USA) (21). Transient transfection with the DNA plasmid was performed using the HilyMax reagent (Dojindo, Kumamoto) in accordance with the manufacturer's recommendations. Briefly, A549 cells cultured in 24-well plates were transfected with the DNA-transfection reagent mixture at 70%–80% confluency. At 24 h after transfection, cells were treated with drugs and harvested for detection of luciferase activity.

Luciferase activity was measured with a luminometer (Lumat LB9507; EG&G Berthold, Bad Wildbad, Germany) using the Dual Luciferase Assay kit (Promega, San Luis Obispo, CA, USA). Co-transfection with phRL-TK (Promega), which expresses *Renilla* luciferase, was performed to enable normalization of data for transfection efficiency.

#### *Statistical analysis*

Differences in the values of the means  $\pm$  S.E.M. among groups were assessed using the Student-Newman-Keuls test. All statistical tests were two-tailed, and  $P < 0.05$  was considered significant.

## **Results**

#### *Effect of GL on goblet cell hyperplasia induced by LPS*

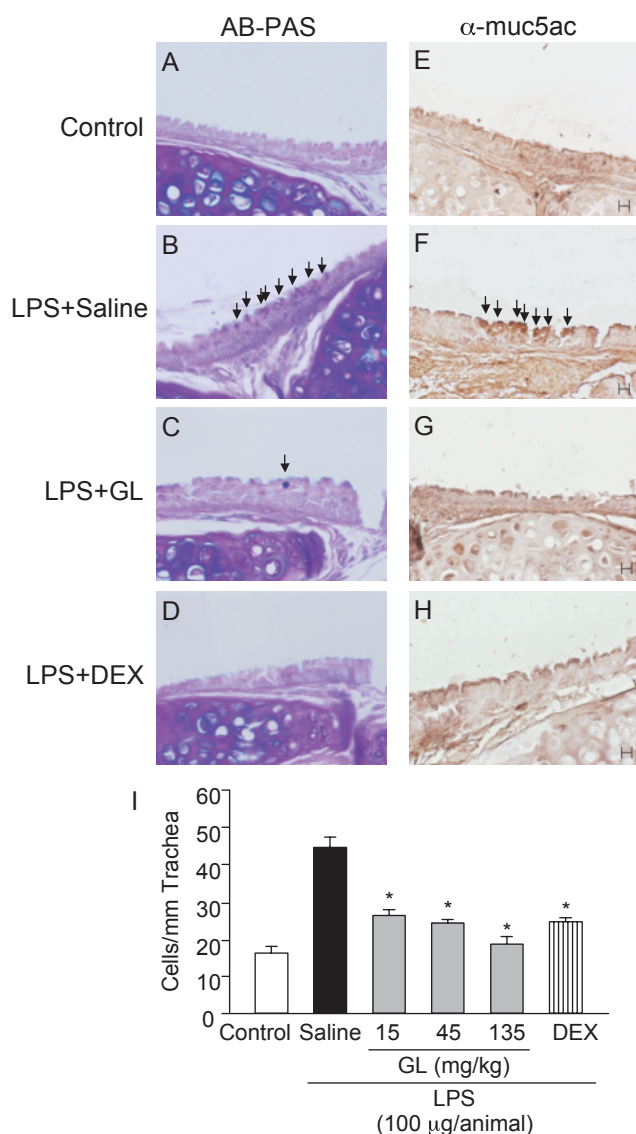
To determine whether GL attenuates mucus hyperproduction, we first examined the effect of GL in a mouse model of goblet cell hyperplasia induced by intratracheal instillation of LPS. Seven days after LPS instillation, morphological changes from tracheal tissue sections were assessed by AB-PAS stain (Fig. 1: A–D). AB-PAS-staining cells were rare in the tracheal epithelium from control mice that had received vehicle instillations. In mice that received LPS, there were prominent AB-PAS-stained cells in the tracheal epithelia. Treatment with GL, as well as DEX, considerably decreased the level of AB-PAS-positive cells induced by LPS. Furthermore, the effect of GL was dose-dependent (15, 45, and 135 mg/kg).

To determine whether the increase in AB-PAS-positive cells was related to *MUC5AC* expression, tracheae from LPS-treated mice were stained with anti-MUC5AC, and *MUC5AC* mRNA expression was assessed by real-time PCR. Consistent with the data of the AB-PAS staining, *MUC5AC*-positive cells are increased in the tracheae of LPS-treated mice and GL considerably inhibited this increase (Fig. 1: E–H). In addition, GL attenuated the increase in *MUC5AC* mRNA expression induced by LPS (Fig. 2), suggesting that GL decreased goblet cell hyperplasia through the inhibition of *MUC5AC* mRNA expression. On the other hand, LPS increased leukocyte infiltration into the lungs at days 1 and 3, as determined by numbers in the BAL fluid, which was prior to the increase in tracheal goblet cells (Supplementary Fig. 1: available in the online version only).

Both GL and DEX inhibited not only goblet cell hyperplasia and *MUC5AC* expression, but also the inflammatory reaction induced by LPS.

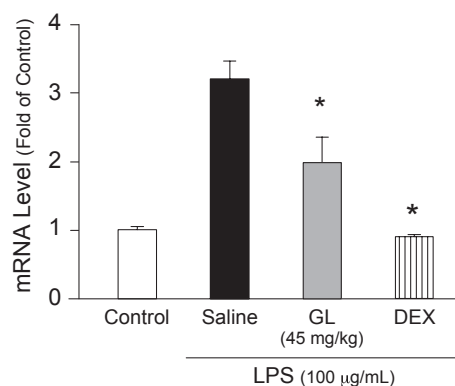
#### *Effect of GL on goblet cell hyperplasia induced by IL-4*

We then examined the effect of GL on mucus hyper-



**Fig. 1.** In vivo effect of GL and DEX on goblet cell hyperplasia and mucus production in LPS-treated mice. Representative photomicrographs of tracheal sections obtained from ICR mice 7 days after intratracheal instillation of LPS (100  $\mu$ g/animal). Tracheal sections stained with AB-PAS were prepared from mice treated with saline (A), LPS and saline (B), LPS and 135 mg/kg of GL (C), or LPS and 1 mg/kg of DEX (D). Arrows indicate goblet cells. Tracheal sections stained with anti-MUC5AC were made from mice treated with saline (E), LPS and saline (F), LPS and GL (G), or LPS and DEX (H). Arrows indicate MUC5AC-positive cells. The number of AB-PAS-positive cells was counted as goblet cells in a 3-mm length of the trachea (I). Results represent the mean  $\pm$  S.E.M. ( $n = 3-4$ ;  $*P < 0.05$ , compared with LPS and saline-treated mice).

production induced by intratracheal instillation of IL-4. In good agreement with previous reports (19, 22), IL-4 increased AB-PAS-positive goblet cells in tracheal epithelium 24 h after the instillation (Fig. 3: A – D), without increasing the number of cells in BAL fluid (Supplemen-



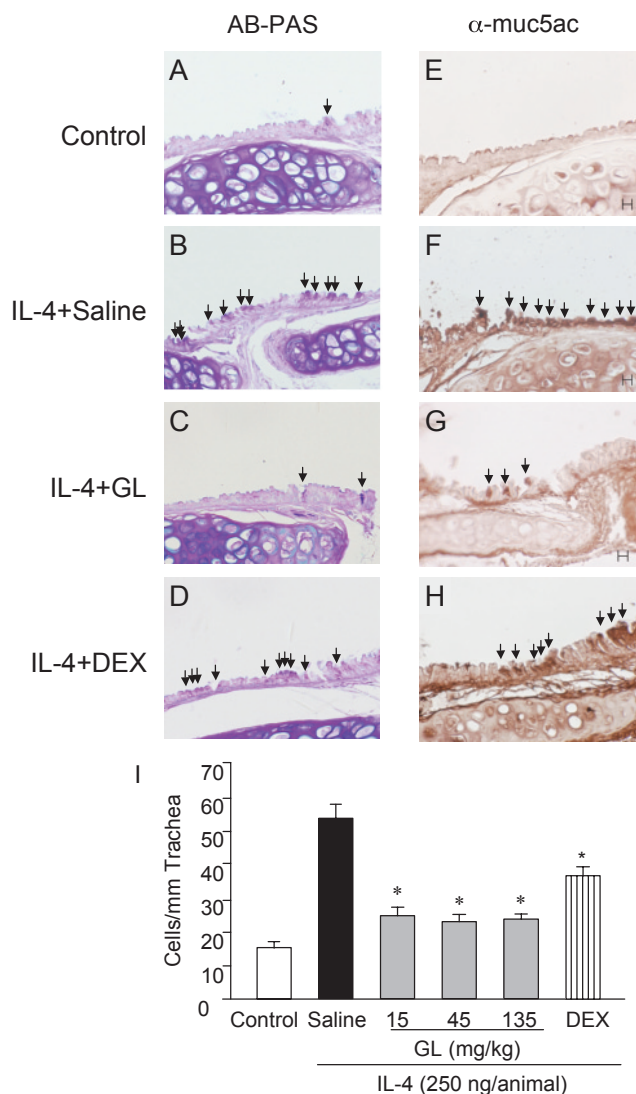
**Fig. 2.** In vivo effect of GL and DEX on increase of *MUC5AC* expression in LPS-treated mice. Total RNA was extracted from lungs 7 days after LPS-treatment. The expression levels of *MUC5AC* mRNA were measured by real-time PCR and normalized with GAPDH. Results represent the mean  $\pm$  S.E.M. ( $n = 3$ ;  $*P < 0.05$ , compared with LPS and saline-treated mice).

tary Fig. 2: available in the online version only). The increase in goblet cells induced by IL-4 was confirmed by the increase in MUC5AC-positive cells by immunohistochemical staining (Fig. 3: E – H) and the increase in *MUC5AC* mRNA expression (Fig. 4). GL (15, 45, and 135 mg/kg, s.c.), as well as DEX (1 mg/kg, s.c.), significantly attenuated the IL-4-induced goblet cell hyperplasia and *MUC5AC* mRNA expression (Figs. 3 and 4). These data suggested that the inhibition of *MUC5AC* mRNA expression by GL and DEX might be independent of the anti-inflammatory effects of these drugs.

#### Effect of GL on *MUC5AC* mRNA and protein expression induced by TGF- $\alpha$

To evaluate the direct effect of GL on mucus production by airway epithelial cells, *MUC5AC* production by NCI-H292 cells was determined. Cells were treated with TGF- $\alpha$  (50 ng/mL) for 24 h, and then *MUC5AC* protein levels in the spent culture media and cell lysates were measured. TGF- $\alpha$  markedly increased the amount of *MUC5AC* protein in both medium and cell lysates compared with those of control cells (Fig. 5). In addition, GL (10, 100, and 1000  $\mu$ M), as well as DEX (0.1  $\mu$ M), significantly inhibited the TGF- $\alpha$ -induced increase in *MUC5AC* in both media and lysates (Fig. 5). The *MUC5AC* mRNA level was also determined by real-time RCR. TGF- $\alpha$  caused a 20-fold increase in *MUC5AC* mRNA levels compared with that in the control (Fig. 6). Co-treatment with GL attenuated the increase in *MUC5AC* mRNA in a concentration-dependent manner (10 – 1000  $\mu$ M). These data suggested that GL inhibited *MUC5AC* production through the inhibition of *MUC5AC* mRNA expression in epithelial cells.

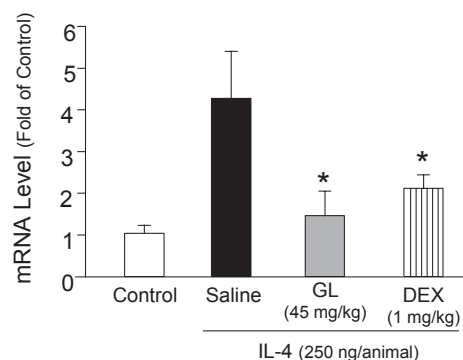




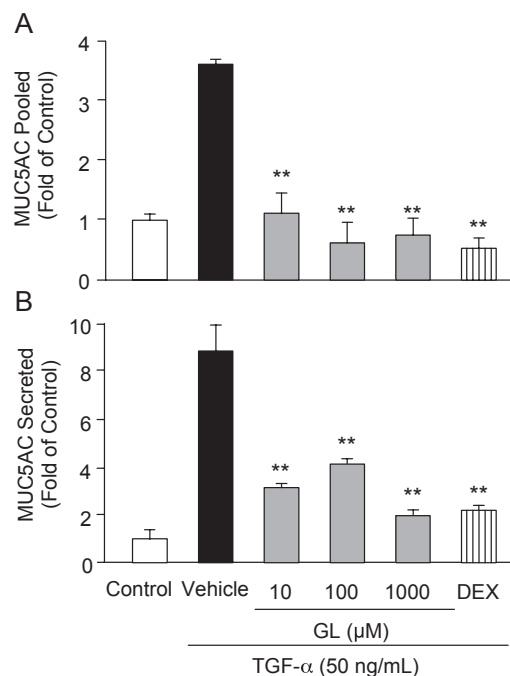
**Fig. 3.** In vivo effect of GL and DEX on goblet cell hyperplasia and mucus production in IL-4-treated mice. Tracheal sections stained with AB-PAS were prepared from mice treated with saline (A), 250 ng/animal of IL-4 and saline (B), IL-4 and 135 mg/kg of GL (C), or IL-4 and 1 mg/kg of DEX (D). Arrows indicate goblet cells. Tracheal sections stained with anti-MUC5AC were made from mice treated with saline (E), IL-4 and saline (F), IL-4 and GL (G), or IL-4 and DEX (H). The number of AB-PAS-positive cells was counted as goblet cells in a 3-mm length of the trachea (I). Results represent the mean  $\pm$  S.E.M. ( $n = 3-4$ ;  $*P < 0.05$ , compared with IL-4 and saline-treated mice).

#### Effect of GL on MUC5AC mRNA transcription induced by TGF- $\alpha$

To examine whether GL inhibited MUC5AC transcription, A549 cells were transfected with a luciferase reporter plasmid containing the human MUC5AC promoter, and the effect of GL was examined. In good agreement with the changes in the levels of MUC5AC protein and mRNA, TGF- $\alpha$  activated the MUC5AC promoter gene

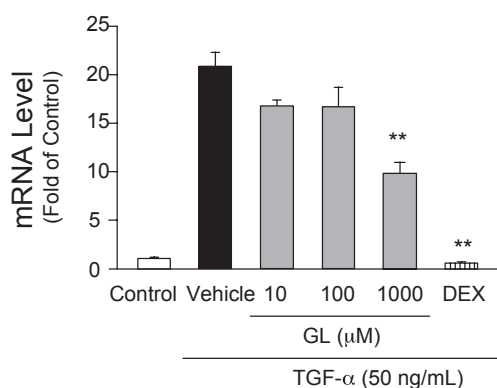


**Fig. 4.** In vivo effect of GL and DEX on the increase in MUC5AC expression in IL-4-treated mice. Total RNA was extracted from trachea 24 h after IL-4 treatment. The expression levels of MUC5AC mRNA were measured as described in Fig. 2. ( $n = 3$ ;  $*P < 0.05$ , compared with LPS and saline-treated mice).



**Fig. 5.** Effect of GL and DEX on MUC5AC protein production and secretion induced by TGF- $\alpha$  in NCI-H2923 cells. Cells were treated with TGF- $\alpha$  (50 ng/mL) and the indicated concentration of GL or DEX for 24 h. The expression levels of MUC5AC in culture media (A) and cell lysates (B) were measured by slot-blot analysis. Results represent the mean  $\pm$  S.E.M. ( $n = 3$ ;  $**P < 0.01$ , compared with TGF- $\alpha$  alone).

(Fig. 7A). GL and DEX significantly attenuated the TGF- $\alpha$ -induced increase in luciferase activity, whereas these drugs did not change the basal promoter activity (Fig. 7A). The effects of GL and DEX on MUC5AC mRNA stability were also determined. NCI-H292 cells were treated with actinomycin D for the indicated times



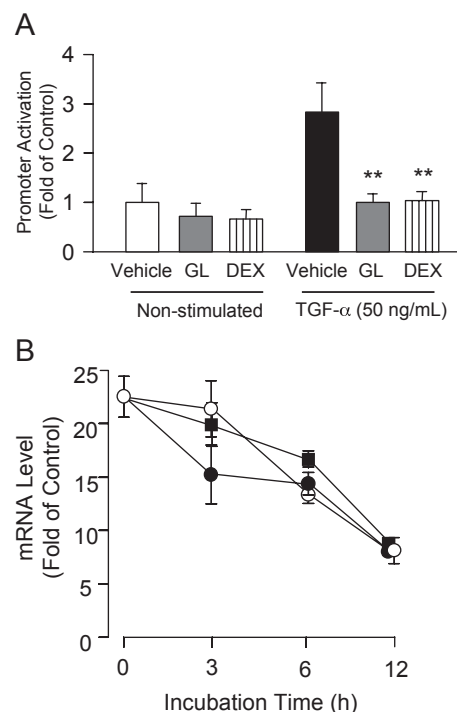
**Fig. 6.** Effect of GL and DEX on *MUC5AC* mRNA expression induced by TGF- $\alpha$  in NCI-H2923 cells. Cells were treated with TGF- $\alpha$  (50 ng/mL) and the indicated concentration of GL or DEX for 24 h, after which total cellular RNA was extracted. The expression levels of *MUC5AC* mRNA were measured by real-time PCR and normalized with GAPDH. Results represent the mean  $\pm$  S.E.M. (n = 3; \*\* $P$  < 0.01, compared with TGF- $\alpha$  alone).

following TGF- $\alpha$  treatment for 12 h. At all of the time points, neither GL nor DEX affected the *MUC5AC* mRNA level (Fig. 7B). These data suggested that GL attenuated the TGF- $\alpha$ -induced increase in *MUC5AC* transcription, but did not affect the stability of *MUC5AC* mRNA.

## Discussion

Airway goblet cell hyperplasia and increase in mucus production are prominent pathophysiological features of chronic inflammatory lung diseases such as COPD, asthma, and cystic fibrosis (1, 23–25). Therefore, in the treatment of chronic airway diseases, pharmacological control of mucus production is important, as well as control of inflammation. We have previously reported that GL inhibits IL-8 expression and NF- $\kappa$ B-dependent transcription in airway epithelial cells and suggested its anti-inflammatory effect (13). Therefore, in the present study we examined the effect of GL on goblet cell hyperplasia and *MUC5AC* production with two different in vivo mouse models. The data clearly indicated that GL, as well as DEX, can inhibit both LPS- and IL-4-induced goblet cell hyperplasia (Figs. 1 and 3). In addition, GL inhibited *MUC5AC* mRNA expression in both models. Therefore, the inhibitory effect of GL on goblet cell hyperplasia may be due to the inhibition of mucus gene expression in airway epithelial cells.

In mice, a single intratracheal instillation of LPS resulted in the massive recruitment of neutrophils to the lung 1 day after the treatment. LPS-induced neutrophilic inflammation subsided on day 7, while the number of goblet cells peaked on day 7. In addition, several anti-



**Fig. 7.** Effect of GL and DEX on TGF- $\alpha$ -induced *MUC5AC* promoter activity and *MUC5AC* mRNA stability. A: A549 cells were transiently transfected with a luciferase reporter construct containing the *MUC5AC* promoter. Cells were then treated with TGF- $\alpha$  (50 ng/mL) combined with GL (1 mM) or DEX (100 nM) for 24 h. Results represent the mean  $\pm$  S.E.M. (n = 3; \* $P$  < 0.01, compared with TGF- $\alpha$  alone). B: NCI-H2923 cells were pre-treated with TGF- $\alpha$  (50 ng/mL) for 12 h, after which cells were incubated with actinomycin D (10  $\mu$ g/mL) (open circle), actinomycin D + GL (1 mM) (closed circle), or actinomycin D + DEX (100 nM) (closed square) for 3, 6, or 12 h. The levels of *MUC5AC* mRNA were measured by real-time PCR, and the mRNA level was represented as the fold of that in nontreated control cells (mean  $\pm$  S.E.M., n = 3; \*\* $P$  < 0.01, compared with actinomycin D alone).

inflammatory drugs have been reported to attenuate mucus production in this model (26–28). Therefore, the goblet cell hyperplasia and mucus hyperproduction may occur as a secondary effect of infiltrating cells. It was predicted that GL would inhibit goblet cell hyperplasia in LPS-treated mice due to its glucocorticoid-like anti-inflammatory effects. In fact, GL and DEX inhibited neutrophil infiltration, as shown by BAL fluid cell numbers, and mRNA expression of KC and TNF- $\alpha$  (Supplementary Fig. 3: available in the online version only). Therefore, we assume that the attenuation of mucus production by GL in this LPS-model is, at least in part, due to its anti-inflammatory effect.

In contrast, IL-4 increased goblet cell numbers and *MUC5AC* expression without changing the number of cells in BAL fluid. Eosinophils were not detected in BAL fluid (data not shown). These data suggested that the

goblet cell hyperplasia and mucus production by IL-4 was not a secondary effect of infiltrated leukocytes. Similar IL-4-induced goblet cell hyperplasia, which was not accompanied with inflammation, was reported by Dabbagh et al. (19). They also demonstrated that epithelial cells constitutively expressed the IL-4 receptor. Therefore, it is possible that IL-4 directly stimulates the differentiation of epithelial cells into goblet cells and mucus hyperproduction. In IL-4-treated mice, GL considerably attenuated goblet cell hyperplasia and MUC5AC expression, suggesting that GL inhibits not only the inflammatory response, but also the direct effect of IL-4 on airway epithelial cells. To confirm this idea, we examined the effect of GL on MUC5AC expression in cultured NCI-H292 cells and demonstrated that GL, as well as DEX, clearly inhibited TGF- $\alpha$ -induced MUC5AC protein and mRNA expression in the cells. We used TGF- $\alpha$  as a mucus inducer because epidermal growth factor receptor (EGFR) stimulation is known to be a common pathway for MUC5AC expression and goblet cell hyperplasia (29–32). Several lines of evidence have shown that in vivo mucus production induced by Th2 cytokines was through EGFR signaling (33, 34). In addition, the inhibition of EGFR is known to attenuate MUC5AC expression induced by LPS, neutrophil elastase, and oxidative stress in cultured epithelial cells in vitro (4–10). Therefore, it is possible that the inhibition of EGFR-mediated MUC5AC expression may be important even for the in vivo effect of GL in both LPS- and IL-4-induced models.

The attenuation of MUC5AC expression by GL was likely to be, at least in part, dependent on the inhibition of *MUC5AC* transcription, which is similar to the effect of DEX. GL, as well as DEX, attenuated the increase in *MUC5AC* mRNA by TGF- $\alpha$  (Fig. 5). In addition, both GL and DEX decreased the *MUC5AC* promoter activity induced by TGF- $\alpha$ , without affecting the stability of *MUC5AC* mRNA (Fig. 6). The inhibitory effect of DEX on *MUC5AC* mRNA transcription is through the activation of glucocorticoid receptors (GRs). Activated GRs translocate from the cytosol to the nucleus and bind to the glucocorticoid responsive element on the *MUC5AC* promoter region, negatively regulating this promoter (1, 35). It is, therefore, reasonable that DEX could attenuate IL-4-induced MUC5AC production (Figs. 3 and 4), although many Th2 actions by Th2 cytokines are known to be insensitive to glucocorticoids. In addition, it is reported that following GR activation DEX can inhibit ERK phosphorylation (36), which is the downstream signaling pathway of EGFR. However, GL did not activate GR assessed by the MMTV promoter activity in our previous study (13). In addition, the GL treatment did not inhibit ERK phosphorylation induced by TGF- $\alpha$  in our

preliminary study (data not shown). Therefore, we assume that the attenuation of MUC5AC by GL may be due to a different mechanism from that of DEX.

In the present study, GL significantly attenuated *MUC5AC* mRNA expression at only the highest concentration (Fig. 6), whereas MUC5AC protein level was decreased by lower concentration of GL (Fig. 5). This different concentration-dependency of GL between MUC5AC mRNA and protein levels might suggest the possibility that GL affects not only *MUC5AC* transcription but also translation. To our knowledge, a regulatory effect of GL on protein translation has not been reported. Further study is needed to define this point.

In conclusion, we have indicated that GL has a glucocorticoid-like inhibitory effect on LPS- or IL-4-induced mucus production in vivo. In addition, it has been shown that GL inhibits mRNA transcription induced by TGF- $\alpha$ , which is thought to be a common stimulator of mucus production in various pathophysiological conditions. Glucocorticoids are useful for decreasing inflammation in diseases such as asthma, cystic fibrosis, and bronchitis, but their clinical effects on mucus hyperproduction have been controversial, especially against Th2 cytokine-induced mucus production. In this study, the inhibitory effect of DEX on IL-4-induced mucus production was considerably less than its inhibitory effect on LPS. The effects of GL may provide a new insight into treating mucus hyperproduction in airway diseases, although the underlying mechanisms should be clarified in further studies.

## Acknowledgments

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