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The human cathelicidin LL-37 enhances airway mucus production in chronic obstructive pulmonary disease

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ABSTRACT

Airway mucus overproduction is a distinguishing feature of chronic obstructive pulmonary disease (COPD). LL-37 is the only member of human cathelicidins family of antimicrobial peptides and plays a central role in many immune and inflammatory reactions. Increasing evidence suggests the involvement of LL-37 in the pathogenesis of COPD. Here, we investigated the effects of LL-37 on airway mucus overproduction in COPD. We observed overexpression of both LL-37 and MUC5AC mucin (a major mucin component of mucus) in airways of COPD patients and found a correlation between them. We showed *in vitro* that LL-37 induces MUC5AC mucin production by airway epithelial NCI-H292 cells in the absence and presence of cigarette smoke extract, with TNF-α converting enzyme (TACE)–EGFR–ERK1/2 pathway and IL-8 required for the induction. Therefore, we concluded that LL-37 enhances the mucus production in COPD airways, thus contributing to the progression of COPD.

1. Introduction

The human airway surface is lined with a layer of mucus produced by secretory epithelial cells, which protects the underlying airway epithelium from exogenous insults including pathogens and harmful particles. Mucins such as MUC5AC are main components of mucus and assist in clearance of inhaled foreign materials [1]. Chronic obstructive pulmonary disease (COPD) is a disease of increasing public health importance and will become the third leading cause of death worldwide by 2030 [2]. Airway mucus overproduction is the predominant pathologic feature of COPD, which contributes to airway obstruction, accelerated decline of lung function and increased mortality of COPD patients. Many stimuli relevant to the development of COPD including cigarette smoking, inflammatory cytokines and certain pathogenic microbes can induce the airway mucus overproduction [2]. Recently, antimicrobial peptides (AMPs) have been implicated in the pathogenesis of COPD [3,4]. AMPs are positive charged, amphiphilic short peptides and have been considered essential components of the innate immune system due to their antimicrobial activity and various biological regulatory functions [5]. AMPs are mainly categorized into two families: defensins and cathelicidins. LL-37 is the only identified member of human cathelicidins family so far, and can be generated by epithelial cells, neutrophils and macrophages in response to pro-inflammatory stimuli including cytokines (e.g. IL-17A and IFN-γ), pathogen-associated molecular patterns (PAMPs) or tissue injury [6]. Besides antimicrobial activity against bacteria, fungi and viruses, LL-37 is involved in the regulation of inflammation, cell proliferation and apoptosis [5]. Several studies have demonstrated high levels of LL-37 in induced sputum and bronchoalveolar lavage fluid from COPD patients [3,4,7]. In addition, our previous study showed a markedly increased presentation of LL-37 in airway epithelium of COPD patients, and demonstrated the ability of LL-37 to induce airway inflammation and alveolar apoptosis which are related to the pathogenesis of COPD [8]. However, the effects of LL-37 on airway mucus overproduction have not been explored.

Airway mucus overproduction is mainly mediated by epidermal growth factor receptor (EGFR) signaling pathway in response to various stimuli including cigarette smoke [9], bacterial exoproducts [10] and inflammatory mediators such as TNF-α and IL-1β [11]. Extracellular signal-regulated kinase (ERK1/2) also plays an important role in mucus induction by cigarette smoke and bacterial exoproducts [12]. Metalloproteinases such as TNF-α converting enzyme (TACE) are also involved in airway mucus overproduction by transactivating EGFR signaling pathway due to their ability of shedding ectodomain of pro-EGFR ligands including TGF-α, amphiregulin and HB-EGF [13]. Interestingly, LL-37 has been reported to activate airway epithelial cells by transactivation of EGFR–ERK1/2 pathway involving certain metalloproteinase [14]. These results suggest that LL-37 may have the ability to stimulate mucus

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production by airway epithelial cells. Moreover, the activated airway epithelial cells by LL-37 can release interleukin-8 (IL-8), a potential chemokine, which is capable of stimulating airway MUC5AC mucin expression [14,15], this may assist in airway mucus induction by LL-37. Therefore, it is reasonable to hypothesize that LL-37 overexpressed in the airways of COPD patients can induce mucus overproduction, and thus contributes to the progression of COPD.

Here, we examined the expression of LL-37 and MUC5AC mucin in induced sputum and small airway epithelium from COPD patients and controls, and determined the correlation between them. Then we investigated *in vitro* the effects of LL-37 on mucus production by airway epithelial cells. We also explored the mechanism responsible for the effects.

2. Materials and methods

2.1. Materials

LL-37 (amino acid sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDF LRNLVPRTES) and scrambled LL-37 (sLL-37, amino acid sequence: RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL) were synthesized by GL Biochem, Shanghai, China. The human MUC5AC-specific ELISA kit (E90756Hu) was purchased from Uscn life science, Houston, USA. The human LL-37-specific ELISA kit (HK321-01) was from Hycult Biotechnology, Uden, the Netherlands. The human IL-8 specific ELISA kit (ab174442) and antibodies against human LL-37 (ab64892) and MUC5AC (ab3649) were from Abcam, UK. The AG1478 (9842), U0126 (9903) and antibodies against ERK1/2 (9102) and pERK1/2 (9101) were from Cell Signaling Technology, Beverly, USA. The neutralizing anti-EGFR Abs (610016) was from BD biosciences, San Joes, USA. The TACE inhibitor TAPI-1 (579051) was from Calbiochem, Darmstadt, Germany. The anti-human CXCL8/IL-8 monoclonal antibody (MAB208) and human recombinant IL-8 (rIL-8, 208-IL) were from R&D System, MN, USA.

2.2. Study subjects

We recruited two groups of subjects undergoing lung resection for a solitary peripheral carcinoma in Shandong University Qilu Hospital: 18 COPD patients (COPD group) and 18 age-matched healthy volunteers with normal pulmonary function (control group). All subjects were in a stable condition and underwent pulmonary function testing before surgery. The diagnosis of COPD was established according to published guideline GOLD (2011). Each individual gave written informed consent after the details of the study were explained. The protocol was approved by the Ethics Committee of Shandong University in Jinan, China.

2.3. Sputum induction and determination of LL-37 and MUC5AC mucin

The sputum was collected from all subjects before surgery and processed as described previously [3]. The levels of LL-37 and MUC5AC mucin in induced sputum were determined using ELISA following the manufacturer's instructions.

2.4. Preparation of lung tissues and immunostaining for LL-37 and MUC5AC mucin

For each subject, two to three randomly selected tissue blocks (sample size 15–25 mm) were taken from the subpleural parenchyma of the lobe obtained in surgery, at least 5 cm far from the border of the tumor to avoid the diseased areas. The tissue samples were processed as described previously [16] and immunostained with antibodies recognizing LL-37 and MUC5AC mucin. Representative images were taken under light microscope (Olympus, Tokyo, Japan) at ×400 magnification. The small airways with an internal diameter less than 2 mm were observed and analyzed for each subject.

2.5. Preparation of CSE

Kentucky standard reference cigarettes 3R4F were used for the preparation of cigarettes smoke extraction (CSE). The cigarettes were conditioned at 22 °C and 60% relative humidity for 48 h before use, and JJD100 single channel smoking machine was used with the smoke of 10 cigarettes withdrawn into 10 ml of D-hanks buffer at a rate of 1 puff/min, then the CSE solutions were sterilized by filtration through a 0.22-μm cellulose acetate sterilizing system. The freshly prepared CSE solutions were diluted with RPMI 1640 immediately before used to treat cells. The final CSE working concentration was expressed as cigarette/ml culture medium (cig/ml).

2.6. Cell culture

The NCI-H292 cells, a human airway epithelial cell line were obtained from American Tissue Culture Collection. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

2.7. Western blot analysis for ERK1/2 phosphorylation

Western blot analysis were performed as described previously [14] using primary antibodies against ERK1/2 and phosphorylated ERK1/2. The ratio of phosphorylated ERK1/2 to total ERK1/2 (pERK/tERK) for each sample was calculated with the utilization of densitometry.

2.8. ELISA for MUC5AC mucin and IL-8 produced by NCI-H292 cells

The levels of MUC5AC mucin in the culture supernatants and cell lysates were determined with a specific ELISA kit and summed for each sample as previously described [13]. The summed total MUC5AC mucin was normalized to total protein in cell lysates and was expressed as μg/mg protein for each sample. The levels of IL-8 released to the culture supernatants were determined with a specific ELISA kit following the manufacturer's instructions.

2.9. Statistical analysis

Data are expressed as mean ± SEM. The Mann–Whitney *U*-test was used for data from study subjects. Chi-square test was used to compare categorical variables. Correlations were assessed by Spearman's rank test. Comparisons of *in vitro* data between multiple treatment groups were performed using ANOVA, and the Bonferroni post-test. A p-value <0.05 was considered significant.

3. Results

3.1. Subject demographic characteristics

The demographic characteristics of two groups of subjects are shown in Table 1. The ratio of smokers to nonsmokers was significant higher in COPD group compared with control group ($p < 0.05$). The forced expiratory volume of predicted (FEV_1%) and FEV_1/forced vital capacity (FVC) ratio were significantly lower in COPD group compared with control group ($p < 0.05$, respectively).

Table 1
Demographic characteristics of subjects.

	Control group (n = 18)	COPD[a] group (n = 18)
Age, years	60.6 ± 5.2	62.5 ± 4.3
Sex, male/female	15/3	16/2
Smokers/non-smokers	6/12	14/4*
FEV_1[b], % predicted	92.1 ± 4.3	65 ± 7.4*
FEV_1/FVC[c], %	81.7 ± 5.2	64 ± 6.3*

[a] COPD, chronic obstructive pulmonary disease.
[b] FEV_1, forced expiratory volume in 1 s.
[c] FVC, forced vital capacity.
* $p < 0.05$ compared with Control group.

3.2. The expression of LL-37 and MUC5AC mucin in COPD airways and correlation analysis

In small airway epithelium, the immunoreactivity of LL-37 was predominantly presented in various bronchial epithelial cells while the immunoreactivity of MUC5AC mucin was mainly localized to the goblet cells within the airway epithelium (Fig. 1A). The expression intensity of both LL-37 and MUC5AC mucin were significantly higher in COPD group compared with Control group ($p < 0.01$, respectively, Fig. 1B and C), and LL-37 was significantly correlated with MUC5AC mucin in Controls ($r = 0.813$, $p < 0.01$, Fig. 1D) and COPD patients ($r = 0.673$, $p < 0.01$, Fig. 1E). Similar results regarding the expression of LL-37 and MUC5AC mucin and correlations were observed in induced sputum (Supplemental Fig. 1).

3.3. LL-37 induces MUC5AC mucin production in NCI-H292 cells

To investigate the effects of LL-37 on mucus production by airway epithelial cells, NCI-H292 cells were treated with various concentrations of LL-37 for different time periods. LL-37 was shown to induce an increase of MUC5AC mucin dose- and time-dependently (Fig. 2A and C). The cell viability was >80% at the highest concentrations of LL-37 (10 μg/ml), which was determined by MTT (Supplemental Fig. 2A). Additionally, sLL-37 was used to stimulate cells. In contrast, the equimolar concentrations of sLL-37 did not increase the MUC5AC mucin production (Fig. 2A and C), indicating the specificity of LL-37 in the process.

As cigarette smoking is the most commonly encountered risk factor of COPD, we treated NCI-H292 cells with LL-37 in the presence of CSE to better model this microenvironment *in vitro*. We focused on the modulation of CSE-induced mucin production by LL-37 and found that CSE-induced MUC5AC mucin production was enhanced by LL-37 in a concentration- and time-dependent manner (Fig. 2B and D). A decreased enhancement of MUC5AC mucin production caused by co-stimulation with LL-37 and CSE at higher concentrations might be attributed to the potential cellular toxicity of combined high doses of stimuli (Fig. 2B). The effect of CSE used in the experiment on cell viability was also determined by MTT (Supplemental Fig. 2B).

3.4. LL-37 activates EGFR-ERK1/2 signaling pathway

As ERK1/2 activation has been reported to be involved in mucus induction, the effect of LL-37 on ERK1/2 activation in NCI-H292 cells was studied. After 15 min stimulation, ERK1/2 activation was most pronounced (Fig. 3A), therefore in additional experiments ERK1/2 activation was determined after 15 min stimulation. LL-37 was shown to induce ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 3B), suggesting the ability of LL-37 to activate ERK1/2. We further investigated whether EGFR is involved in LL-37-induced ERK1/2 activation. Pretreatment cells with EGFR tyrosine kinase inhibitor AG1478 and neutralizing anti-EGFR Abs for 1 h significantly inhibited LL-37-induced ERK1/2 activation (Fig. 3C). These results indicated a role of EGFR in LL-37-induced ERK1/2 activation. Additionally, as TACE has been demonstrated to cleave EGFR ligands from their membrane-anchored forms and thus to be involved in EGFR transactivation in this way, the effect of the TACE inhibitor TAPI-1 was evaluated. TAPI-1 was shown to effectively inhibit LL-37-induced ERK1/2 activation (Fig. 3C).

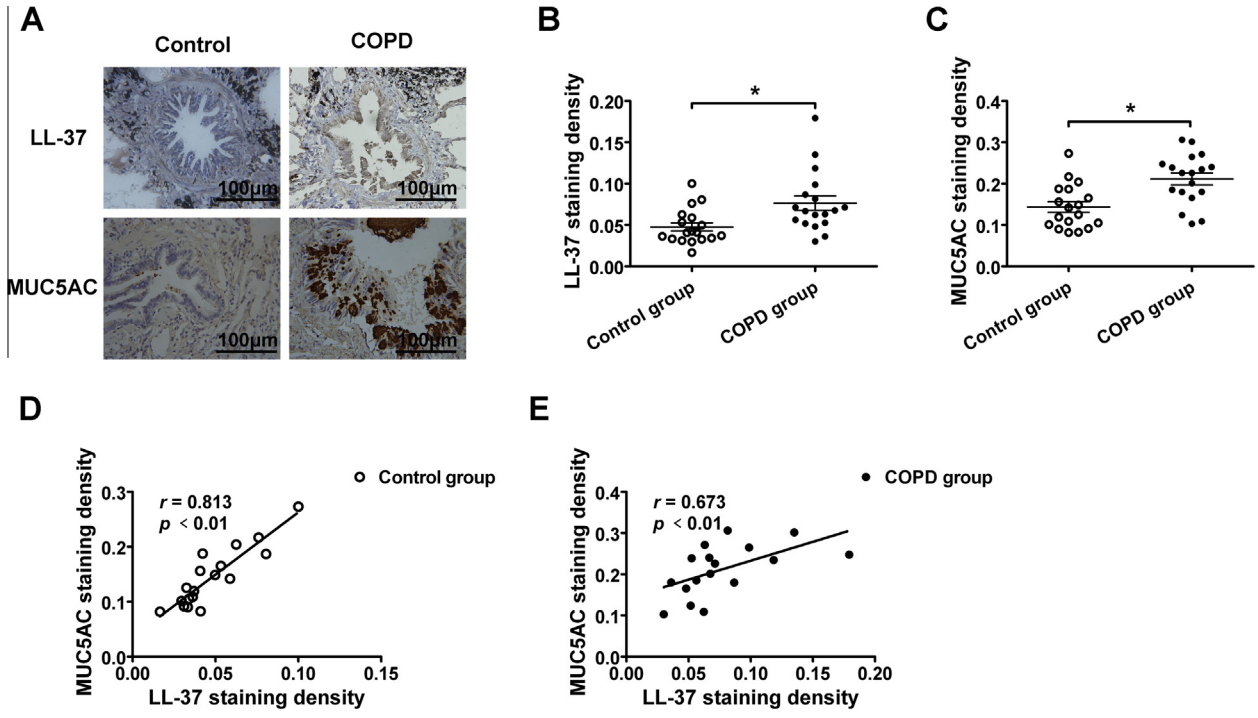

Fig. 1. The expression of LL-37 and MUC5AC mucin in the airway epithelium and correlation analysis. (A) The immunostaining of LL-37 and MUC5AC mucin in small airway epithelium. (B and C) The mean staining density of LL-37 (B) and MUC5AC mucin (C). (D and E) The correlation between LL-37 and MUC5AC mucin in airway epithelium of Controls (D) and COPD patients (E). Data are expressed as mean ± SEM. *$p < 0.01$.

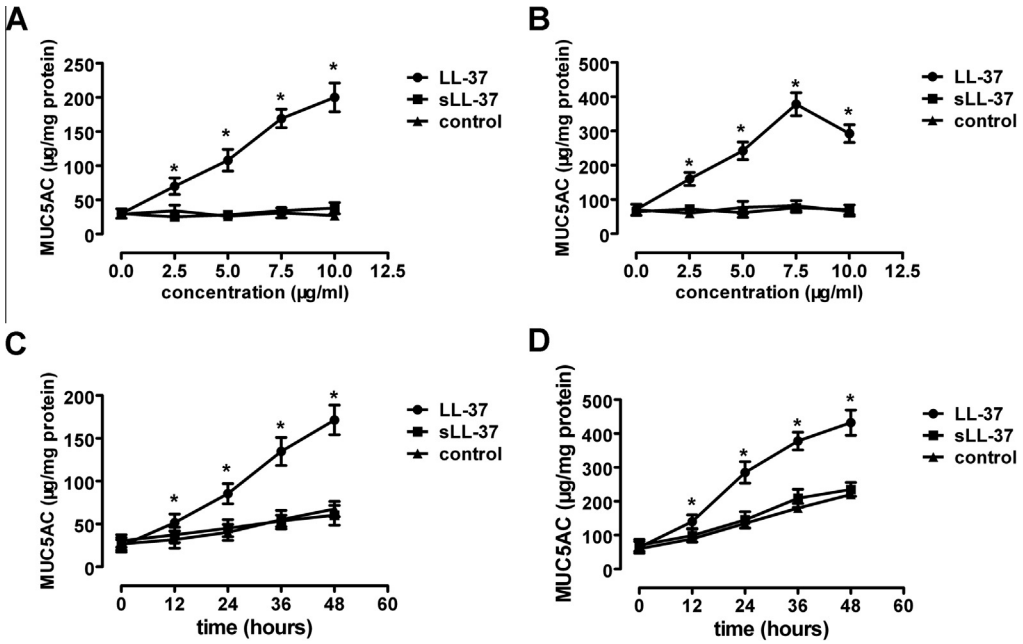

Fig. 2. LL-37 induces MUC5AC mucin production in NCI-H292 cells. (A and B) Cells were treated with various concentrations of LL-37 and scrambled LL-37 (sLL-37) for 24 h in the absence (A) and presence (B) of CSE (0.005 cig/ml). (C and D) Cells were treated with LL-37 (5 μg/ml) and sLL-37 (5 μg/ml) for different time periods in the absence (C) and presence (D) of CSE (0.005 cig/ml). The MUC5AC mucin in cell lysates and culture media were determined by ELISA and summed, and then normalized to total protein in cell lysates. Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.01$ compared with untreated controls.

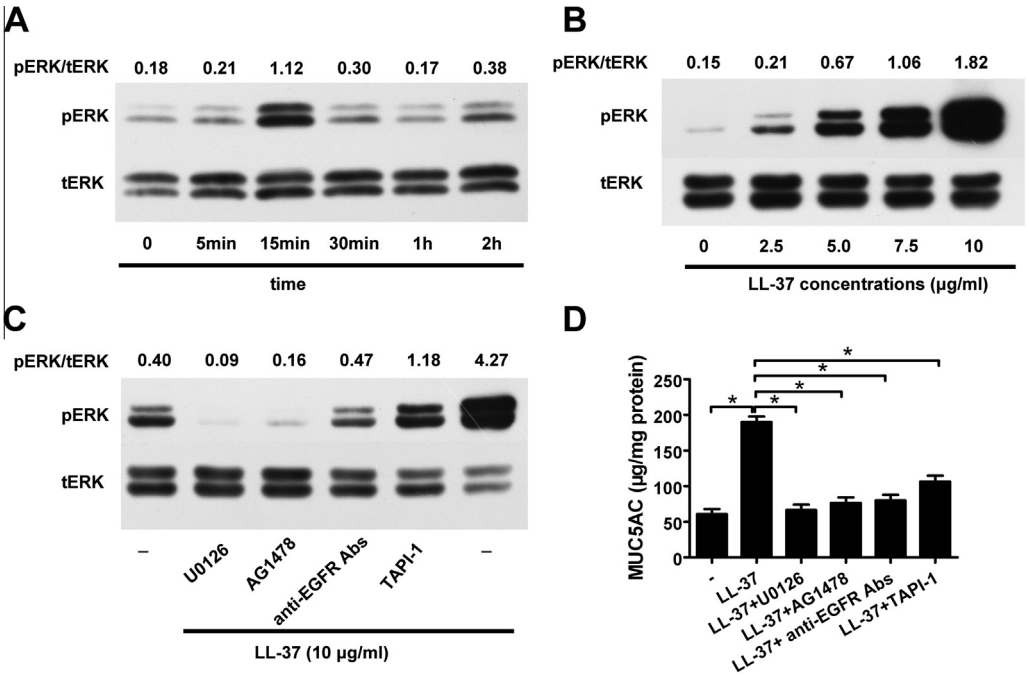

Fig. 3. Transactivation of EGFR–ERK1/2 pathway involving TACE is required for MUC5AC mucin induction by LL-37. (A) NCI-H292 cells were treated with LL-37 (5 μg/ml) for different time periods. (B) NCI-H292 cells were treated with various concentrations of LL-37 for 15 min. (C) NCI-H292 cells were preincubated with U0126 (25 μM), AG1478 (1 μM), anti-EGFR Abs (2 μg/ml) and TAPI-1 (3 μM) for 1 h before treated with LL-37 (10 μg/ml) for 15 min. Cell lysates were collected for the determination of phosphorylated (pERK) and total EGFR (tERK) levels by Western blot analysis. The ratio of pERK/tERK for each sample was calculated and shown at the top of each lane. Data are representative of three individual experiments. (D) NCI-H292 cells were preincubated with U0126 (25 μM), AG1478 (1 μM), anti-EGFR Abs (2 μg/ml) and TAPI-1 (3 μM) for 1 h before LL-37 (10 μg/ml) was added, after 24 h stimulation, the total MUC5AC mucin in cell lysates and culture media were determined as described above. Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.01$.

3.5. LL-37-induced MUC5AC mucin production is mediated via EGFR-ERK1/2 pathway and requires TACE activity

To further investigate the involvement of EGFR-ERK1/2 pathway in LL-37-induced MUC5AC mucin production, NCI-H292 cells were incubated with inhibitors of separate signaling molecules for 1 h before adding LL-37, and after 24 h stimulation, total MUC5AC mucin production in cell culture supernatants and cell lysates were determined (Fig. 3D). The MEK inhibitors U0126 completely inhibited LL-37-induced MUC5AC mucin production,

suggesting that ERK1/2 is centrally involved in LL-37-induced MUC5AC mucin production. The EGFR tyrosine kinase inhibitors AG1478 and neutralizing anti-EGFR Abs effectively blocked the MUC5AC mucin production, indicating a role of EGFR in MUC5AC mucin induction by LL-37. All these results indicated that EGFR–ERK1/2 pathway is required for the LL-37-induced MUC5AC mucin production. Moreover, to study the involvement of TACE in LL-37-induced MUC5AC mucin production, the effect of the TACE inhibitor TAPI-1 was studied. Pretreatment cells with TAPI-1 effectively inhibited MUC5AC mucin induction, suggesting the involvement of TACE in LL-37-induced MUC5AC mucin production.

3.6. IL-8 is involved in LL-37-induced MUC5AC mucin production by NCI-H292 cells

To further dissect the signaling pathway involved in LL-37-induced MUC5AC mucin production, we investigated the role of IL-8 in the process. NCI-H292 cells were treated with various concentrations of LL-37 for different time periods. LL-37 was shown to induce IL-8 production in a concentration- and time-dependent manner in the absence and presence of CSE (Fig. 4A and B). Inhibitors of metalloproteinase TACE and EGFR–ERK1/2 pathway significantly inhibited LL-37 induced IL-8 production (Fig. 4C), indicating that the MUC5AC mucin and IL-8 induction by LL-37 share similar upstream signaling pathway. Furthermore, exogenous IL-8 stimulated the MUC5AC mucin production by NCI-H292 cells and pretreatment cells with anti-IL-8 neutralizing Abs effectively inhibited LL-37-induced MUC5AC mucin production (Fig. 4D). These results suggest that IL-8 induced by LL-37 is involved in the MUC5AC mucin production by airway epithelial cells to enhance the process.

4. Discussion

In the present study, we investigated the effects of LL-37 on airway mucus overproduction in COPD patients for the first time. Our *in vivo* data showed that COPD patients have markedly increased expression of LL-37 and MUC5AC mucin in small airways compared with healthy individuals, and we observed significant correlations between the two parameters. We also found *in vitro* that LL-37 stimulates MUC5AC mucin production in the presence and absence of CSE in cultured NCI-H292 cells, with TACE–EGFR–ERK1/2 pathway involved in the process. In addition, we found that IL-8 induced by LL-37 is involved in LL-37-induced MUC5AC mucin to enhance this process. Altogether, these results suggest that overexpressed LL-37 in small airways of COPD patients may induce the mucus overproduction, thus contributing to the progression of COPD.

Excessive airway mucus production is the hallmark of COPD and contributes to the disease progression [2]. In consistent with many previous findings, our data showed a significant increase of MUC5AC mucin in small airways of COPD patients compared with healthy volunteers. In this study, a majority of the COPD patients were smokers, and cigarette smoking may be responsible for the excessive expression of MUC5AC mucin as it is the principal causative factor of COPD and can induce airway inflammation accompanied with mucus overproduction [2].

Antimicrobial peptides are essential components of the respiratory innate immunity. LL-37 the only member of human cathelicidin, is mainly produced by airway epithelial cells and neutrophils in respiratory tract [17]. Besides potential antimicrobial activity, LL-37 has been reported to play pivotal roles in regulating the immune inflammation response [5]. Several studies have focused on the effects of LL-37 in inflammatory airway diseases

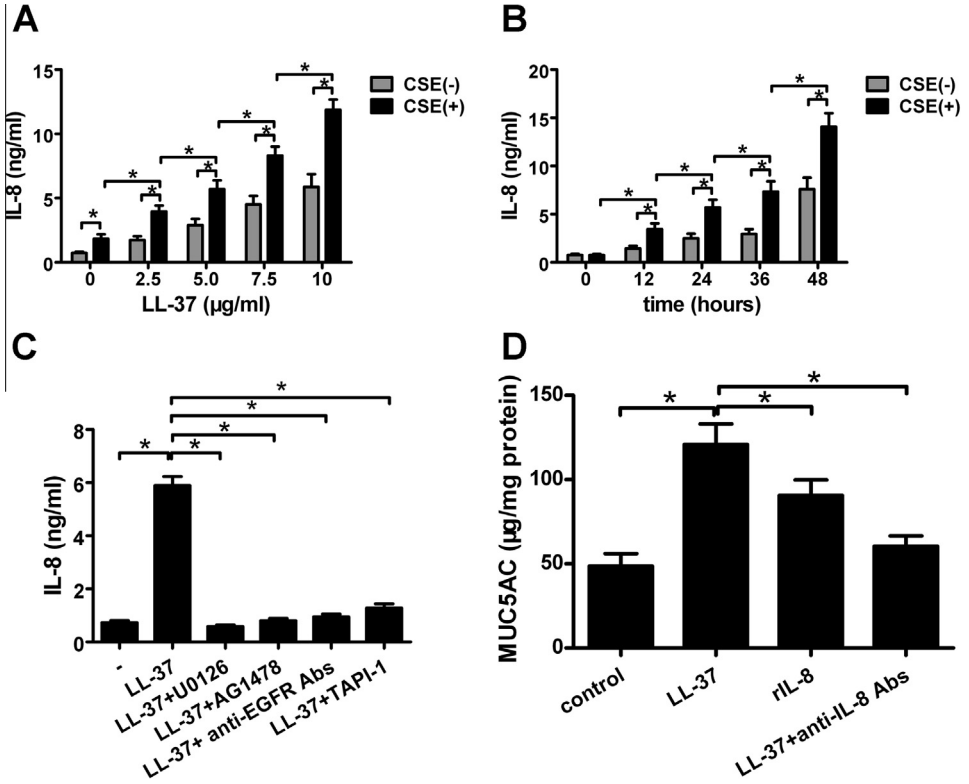

Fig. 4. IL-8 induced by LL-37 enhances MUC5AC mucin induction in NCI-H292 cells. (A) Cells were treated with various concentrations of LL-37 for 24 h in the absence and presence of CSE (0.005 cig/ml). (B) Cells were treated with LL-37 (5 μg/ml) for different time periods in the absence and presence of CSE (0.005 cig/ml). (C) Cells were preincubated with U0126 (25 μM), AG1478 (1 μM), anti-EGFR Abs (2 μg/ml) and TAPI-1 (3 μM) for 1 h and then treated with LL-37 for 24 h. The IL-8 in culture media was determined using ELISA. (D) Cells were treated with LL-37 (5 μg/ml), rIL-8 (10 ng/ml) and LL-37 plus anti-IL-8 Abs (5 μg/ml) for 24 h, the total MUC5AC mucin in cell lysates and culture media were determined as described above. Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.01$.

including COPD. Elevated sputum LL-37 levels in COPD patients have been reported [3,4], and previous work by our group demonstrated increased LL-37 levels both in induced sputum and in airway epithelium of COPD patients [8]. Also, LL-37 was found increased in the BALF and EIF of mild to moderate COPD patients [7]. In this study, our results from COPD patients are in accordance with the previous findings, which further confirmed the involvement of LL-37 in the development of COPD. We analyzed the association between LL-37 and MUC5AC mucin expressed in induced sputum and airway epithelium of all the subjects, and found close correlations between the two parameters. Then we found *in vitro* that LL-37 induces MUC5AC mucin production by airway epithelial NCI-H292 cells, which supported the correlation between LL-37 and MUC5AC mucin observed *in vivo*. However, the mechanism by which LL-37 induces MUC5AC mucin was unclear.

Studies have shown that ERK/MAPK cascade is involved in mucus overproduction induced by various stimuli including cigarette smoke and its components [9], cytokines such as TNF-α and IL-1β [11], and microbial exoproducts [10,13]. Here we demonstrated that LL-37-induced MUC5AC mucin production is at least partially mediated via ERK1/2 activation, as shown by the ability of the MEK inhibitor U0126 to inhibit LL-37-induced MUC5AC mucin production. EGFR is also involved in ERK1/2 activation and airway mucus overproduction in response to multiple stimuli [9–11,18]. In the present study, the EGFR seems to be involved in LL-37-induced MUC5AC mucin production because the EGFR tyrosine kinase inhibitor AG1478 effectively inhibits the process. In addition to the involvement of EGFR and ERK1/2, we also found that metalloproteinase activity is required for LL-37-induced MUC5AC mucin production, as we observed that the metalloproteinase TACE inhibitor TAPI-1 inhibits the process. TACE was reported to be implicated in mucus production stimulated by CSE [9] and neutrophil elastase [19] in airway epithelial cells. It is responsible for the ectodomain shedding of membrane-anchored EGFR ligands such as TGF-α, amphiregulin and HB-EGF, which potentially bind to and activate EGFR [13]. In addition, we observed that LL-37-induced MUC5AC mucin production is inhibited by neutralizing anti-EGFR Abs which was used to block the ligand binding sites of EGFR. These results suggest that the LL-37-induced activation of TACE may increase MUC5AC mucin production from bronchial epithelial cells via transactivation of EGFR–ERK1/2 cascade.

Considering the fact that airways of most COPD patients are repetitively exposed to cigarette smoke, we treated the bronchial epithelial cells with LL-37 in the presence of CSE to model the *in vivo* microenvironment. We found that co-stimulation of LL-37 and CSE dose- and time-dependently increases the MUC5AC mucin production with regards to LL-37. As discussed above, cigarette smoke can induce MUC5AC mucin production by TACE and EGFR signaling pathway, our data indicate that LL-37 and CSE may cooperate with each other to enhance the MUC5AC mucin induction via the similar pathway. Notably, the decreased MUC5AC mucin induction by LL-37 at high (supraphysiologic, more than 2–5 μg/ml detected in healthy airway mucosa) concentration in the presence of CSE may be attributed to cell death caused by LL-37 and CSE. Both LL-37 and CSE possess the ability to induce injury or apoptosis of airway epithelial cells [2,8,20], and the epithelial cells are sensitive to high concentrations of LL-37 encountered during inflammation or injury [20], therefore, concurrent exposure to CSE and high concentration of LL-37 may cause more cell death.

In the present study, we also highlighted the role of IL-8 in LL-37 induced MUC5AC mucin production. As a potential pro-inflammatory mediator and chemokine, IL-8 is deeply involved in the pathogenesis of COPD [2]. Here we showed that IL-8 can be induced by LL-37 in the absence and presence of CSE. Moreover, we found that IL-8 possesses the ability to stimulate MUC5AC mucin production, and that LL-37-induced MUC5AC mucin production can be blocked by neutralizing anti-IL-8 Abs which bind to IL-8 induced by LL-37 in the culture system and thus prevent the downstream effects of IL-8. These results suggest that IL-8 induced by LL-37 is directly involved in LL-37-induced MUC5AC mucin production to help enhance this process.

In summary, we observed close correlations between LL-37 and MUC5AC mucin in COPD airways, and found enhancement effects of LL-37 on airway epithelial MUC5AC mucin production with TACE–EGFR–ERK1/2 pathway and IL-8 involved. We concluded that LL-37 overexpressed in small airways of COPD patients induces the mucus overproduction, and thus contributes to the progression of COPD.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.074.

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