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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).