hCLCA1 DNA Vaccine Suppresses Cell Hyperplasia and Mucin Expression of Goblet Cells in vitro

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Key Words

Asthma · Calcium-activated chloride channel 1 · DNA vaccine · Goblet cell · Mucus

Abstract

Background: Excessive airway mucus secretion is a remarkable trait of asthma. Mucus overproduction mainly resulted from an increase in goblet cell numbers, which causes considerable damage to health. However, effective therapeutic treatments are still lacking for mucus hypersecretion. Human calcium-activated chloride channel 1 (hCLCA1) has been identified to be predominantly responsible for mucus hypersecretion. **Objectives:** In this study, we investigated the effects of an hCLCA1 DNA vaccine on the control of mucus production and goblet cell proliferation using an in vitro model goblet cell line (NCI-H292). **Methods:** The effect of the hCLCA1 DNA vaccine on cell viability and proliferative activity of NCI-H292/hCLCA1 was analyzed by electron microscopy, MTT assay, and flow cytometry. Expression of mucins and MUC5AC, a major member of the mucin gene family in airway goblet cells, was assessed under hCLCA1 DNA vaccine challenges by periodic acid-Schiff staining, quantitative real-time PCR and Western blot, respectively, and the expression profile of granulocyte-macrophage colony-stim-

ulating factor (GM-CSF), a critical cytokine in airway inflammation, was also examined by real-time PCR and immunocytochemistry. **Results:** Results showed that hCLCA1 overexpression caused high cell proliferation and mucin expression, whereas the hCLCA1 DNA vaccine could effectively reverse these abnormal effects. In addition, GM-CSF expression was highly induced by hCLCA1 overexpression and efficiently suppressed by hCLCA1 DNA vaccine. **Conclusions:** These results illustrate that the hCLCA1 DNA vaccine effectively inhibits cell hyperplasia and mucin gene expression of goblet cells, suggesting that the hCLCA1 DNA vaccine has potential value in the treatment of human asthma.

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Introduction

Excessive airway mucus secretion is a remarkable trait of chronic airway diseases including asthma, a chronic inflammatory disease caused by pathological changes of airway epithelial cells (AECs). Mucus overproduction arising from an increase in goblet cell numbers results in

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airway obstruction, loss of lung function, and even asthmatic death [1]. To date, there is still a lack of effective therapeutic treatment for mucus hypersecretion.

For many years, infiltration of eosinophils and secretion of various cytokines and chemical mediators were associated with asthma pathology, and airway inflammation and remodeling in both the central and small airways were characteristics of asthma [2, 3]. However, little is known about the underlying pathological gene expression. In recent years, increasing evidence has clarified that calcium-activated chloride channel (CLCA) proteins are tightly associated with asthma and are specifically expressed in goblet cells in the epithelium [4, 5]. To date, bovine, murine, human, and porcine isoforms of the CLCA family have been found [6, 7]. Orthologs of the CLCA family, which originally function as chloride channels, are proposed to play critical roles in diverse cellular processes, including cell adhesion, apoptosis, cell cycle control, tumorigenesis, and metastasis [7], and also some respiratory diseases such as asthma [8] and cystic fibrosis [9]. Previous studies have revealed that goblet cell hyperplasia and mucus hypersecretion are tightly associated with CLCA proteins. Of the CLCA family, human CLCA1 (hCLCA1) and its mouse ortholog CLCA3 (mCLCA3) have been particularly implicated in playing crucial roles in the pathogenesis of asthma [8, 10].

As mCLCA3 and hCLCA1 share 75% homology, the study of mCLCA3 in a mouse model could provide significant insights for hCLCA1 in a human study [11]. Knockdown of mCLCA3 in a mouse model of allergic asthma resulted in the inhibition of goblet cell metaplasia and airway hyperreactivity, while mCLCA3 overexpression caused an increase in the goblet cell number [8]. Similarly, in pulmonary inflammation induced by LPS challenge, an mCLCA3^{-/-} mouse model exhibited milder goblet cell hyperplasia and mucus production compared with the wild-type control [12]. However, some studies suggested that CLCA family members might have diverse functional activities and more than 1 family member mediates mucous cell metaplasia as mCLCA3 gene knockout did not affect mucous cell metaplasia and airway hyperreactivity [13]. Robichaud et al. [14] also demonstrated that mCLCA3^{-/-} mice subjected to allergic challenges or IL-13 administration showed similar goblet cell metaplasia and airway inflammation as the wild type. It seemed that members of the CLCA family may have functional complementation such as mCLCA5 [13]. Thus, the precise role of mCLCA3 in mice airway inflammation needs to be further investigated and verified. mCLCA3 was

found in mucin granule membranes of the respiratory tract, suggesting that it was involved in mucin storage or release [15]. Mucin expression was highly upregulated by overexpression of mCLCA3 or hCLCA1 in the human mucoepidermoid cell line NCI-H292 [8, 10]. Mucins, the predominant component of mucus, are glycoproteins produced by AECs. Mucins are a group of membrane-associated or secreted proteins which comprise about 20 members in humans [16, 17]. Among the mucin gene family, the secreted mucin MUC5AC is specifically and highly expressed and inducible in airway goblet cells under environmental challenges, suggesting a critical role in respiratory diseases [18, 19].

Numerous studies raised the possibility that CLCA proteins function as signaling molecules rather than as anion channels that control mucin production. Both mCLCA3 and hCLCA1 were reported to be cleaved into two subunits (a ~90-kDa N-terminal and ~30-kDa C-terminal products) that are secreted soluble proteins [20, 21] and Yurtsever et al. [22] have demonstrated that CLCA family proteins have self-cleavage as well as cross-cleavage zinc-dependent proteolytic activity which unmasked the N-terminal fragment of CLCA to activate CLCA-mediated chloride transport. Recent studies demonstrated that the secreted hCLCA1 and mCLCA3 had zinc-dependent autoproteolytic activity that might cleave other proteins [23, 24]. In the process of asthma, AECs are able to produce numerous cytokines in an autocrine or paracrine manner, which in turn regulates asthma pathology. In vitro studies demonstrated that CLCA levels were upregulated by Th2 cytokines, including interleukin (IL)-4, IL-9, and IL-13, indicating that CLCA could be regulated by signal transmission [25, 26].

As hCLCA1 dysregulation is highly correlated with goblet cell hyperplasia and mucus hypersecretion, inhibiting expression of hCLCA1 in airway epithelium of asthma patients may be beneficial for asthma therapy. Thus, hCLCA1 is a functional unit of asthma involving goblet cells, and thus a potential target for the prevention and treatment of asthma. Administration of specific inhibitors of chloride channels, e.g. niflumic acid, talniflumate, and MSI-2216, was able to block hCLCA1 expression by suppressing JAK/STAT6 activation [26]. As hCLCA1 was not considered as a chloride channel, these inhibitors suppressed hCLCA1 expression implying that hCLCA1 was associated with chloride channels through unknown pathways such as JAK/STAT6. Recently, it has been demonstrated that hCLCA1 does not form Ca²⁺-dependent Cl⁻ channels but modulates calcium-dependent chloride currents in the HEK293 and NCI-H522 expression sys-

tems [27]. Due to safety aspects of these inhibitors, they are currently not available for asthma treatment. Presently, with advancing molecular technology, DNA vaccine therapy has been developed and widely used in human clinical trials against various infectious diseases and cancer. DNA vaccination provides an attractive technique to protect an organism against disease through activation of the immune system and antibody production. Thus, DNA vaccines have unique advantages and are worth pursuing.

In this study, we evaluated the effect of hCLCA1 DNA vaccine treatment on the control of goblet cell hyperplasia and mucin production in vitro. We first established the NCI-H292/hCLCA1 cell line via introducing constructed expression vectors pIRES2-EGFP/hCLCA1 into the NCI-H292 cell line. NCI-H292/hCLCA1 cells showed higher proliferation capability and synthesis activity than native cells using electron microscopy, MTT viability assay, and flow cytometric analysis. Furthermore, the expression levels of MUC5AC and granulocyte-macrophage colony-stimulating factor (GM-CSF) were also elevated. These abnormal effects induced by hCLCA1 overexpression could be significantly inhibited by hCLCA1 DNA vaccine treatment. Taken together, these results indicate that the hCLCA1 DNA vaccine might be an effective strategy for asthma therapy.

Materials and Methods

Design

This was a basic study evaluating the effects of hCLCA1 DNA vaccine on the treatment of asthma using a goblet cell model. An in vitro goblet cell model was established by introducing the constructed expression vector pIRES2-EGFP/hCLCA1 into the NCI-H292 cell line.

Animals

Male BALB/c mice between 6 and 8 weeks of age were purchased from the Experimental Animal Center of Shaanxi Province, China. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Fourth Military University. Mice were kept under standard conditions at room temperature (RT), dark-light cycles and humidity with food and water available ad libitum. All efforts were made to minimize suffering.

Antiserum Preparation

SecTag2-B/hCLCA1 vectors were constructed as previously described [28]. Briefly, the hCLCA cDNA1 fragments in the plasmid pcDNA3.1+/hCLCA1 (gifts from Dr. R.C. Levitt, Genaera) were released by digestion with the restriction enzymes *NheI* and *XhoI* (Takara, Tokyo, Japan). The sense primer 5'-CAAGCT-TATGGGGCCATTAAAGAGTTC-3' and the antisense primer

5'-CTAGGCTATTGACAGCTGCA-3' were used to amplify the hCLCA1 DNA fragments with digestion sites *HindIII* and *XhoI*. The amplified gene was cloned into the pSecTag2-B expression vector (Invitrogen, Carlsbad, Calif., USA) with T7 promoter (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000354180) to construct an hCLCA1 DNA vaccine confirmed by sequencing. Ten male BALB/c mice were injected with 50 µl 0.5% bupivacaine in the quadratus femoris muscle of the hind limb. After 24 h, 50 µl of pSecTag2-B/hCLCA1 DNA was injected in the same position. The mice were vaccinated every 2 weeks over a period of 8 weeks. Mouse tails were cut every 2 weeks after vaccination, and 100 µl of blood were collected and centrifuged (2,000 rpm). The serum was stored frozen and prepared for the following experiments. Mice vaccinated with control plasmid SecTag2-B served as controls.

Construction of Eukaryotic Expression Plasmids and Transfection

The hCLCA1 cDNA fragments in the pcDNA3.1+/hCLCA1 constructs, a gift from Dr. R.C. Levitt, were released by digestion with the restriction enzymes *NheI* and *XhoI* (Takara). The fragments with restriction sites *NheI* and *XhoI* were subcloned into the plasmid pIRES2-EGFP (Clontech, Palo Alto, Calif., USA). The recombinant construct was named pIRES2-EGFP/hCLCA1.

NCI-H292, a human pulmonary mucopidermoid cell line, was continuously cultured in DMEM (GibcoBRL, Gaithersburg, Calif., USA) supplemented with 10% calf serum at 37°C and 5% CO₂. The pIRES2-EGFP/hCLCA1 (0.8 µg) plasmid was mixed with DMEM without serum to a total volume of 50 µl and incubated at RT for 5 min. Lipofectamine 2000 (2.0 µl; GibcoBRL) was mixed with DMEM without serum, which was then mixed with the above plasmid mixtures and incubated at RT for 20 min. The final mixtures were incubated with NCI-H292/hCLCA1 cells for 6 h. Then, the old medium was removed and new DMEM with 10% calf serum was added. After 72 h, cells were cultured with neomycin (G418; 200 g/l) for 4 weeks to obtain a cell line constitutively expressing NCI-H292/hCLCA1. Fluorescence was investigated under an Olympus fluorescence microscope (Olympus Optical, Tokyo, Japan). For the experiments, cells were divided into 4 groups: C1, NCI-H292 cells with control serum treatment (antisera against pSecTag2-B); C2, NCI-H292 cells with hCLCA1 immune serum (antisera against pSecTag2-B/hCLCA1) treatment; H1, NCI-H292/hCLCA1 cells with normal serum treatment, and H2, NCI-H292/hCLCA1 cells with hCLCA1 immune serum treatment.

Western Blot Analysis

Cultured cells were collected and lysed with a lysis buffer containing 1% NP-40 to obtain the protein samples. A total of 20–30 µg of protein were fractionated by 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was treated using the following procedure with shaking and blocking at RT with 2% nonfat dry milk in Tris-buffered saline (TBS) for 1 h followed by incubation in primary antibodies (goat polyclonal anti-mouse CLCA1 antibody, Santa Cruz Biotechnology, Santa Cruz, Calif., USA, and rabbit monoclonal anti-mouse MUC5AC antibody, Abcam, Cambridge, UK) diluted in block buffer (1:10,000) at 4°C overnight and washed three times with TBS and Tween (TBST; 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 10 min

each time at RT. Subsequently, the membrane was incubated in peroxidase-conjugated secondary antibody goat anti-rabbit or horse anti-goat IgG (Boster, Wuhan, China; diluted 1:3,000 in blocking buffer) for 1 h. After washing three times with TBST and once with TBS each for 10 min, 1 ml of 4-chloro-1-naphthol as a horseradish peroxidase substrate with 9 ml of TBS and 6 μ l of H_2O_2 was used for visualizing the target protein in the dark for 5–30 min.

Electron Microscopy

Cells were digested by trypsin (0.15%) and centrifuged at 8,000 rpm for 10 min. The supernatant was removed and the remaining cells were fixed with 2.5% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.05 M) for 2 h. Then, samples were fixed in 1% aqueous OsO_4 for 2 h at 4°C followed by washing in phosphate buffer. Cells were stained in 2% aqueous uranyl acetate for 2 h at 20°C, subsequently dehydrated in a series of graded ethanol, and finally embedded in Epon/Araldite. Samples were cut on an ultramicrotome (RMC MT-7; RMC, Tucson, Ariz., USA) into semi- and ultrathin sections. Ultrathin sections were examined and photographed with a Jeol JEM1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

MTT Assay

Cells were plated in 96-well plates and cultured under regular conditions until they reached the desired confluence. Mouse immune serum diluted 1:200 was added, and cells were incubated at 37°C and 5% CO_2 for 48 h. Then, culture medium was replaced with fresh medium containing MTT (5 mg/ml in PBS, 200 μ l/well; Sangon, Shanghai, China) and incubated with cells for additional 4 h. Formazan was dissolved in DMSO (150 μ l/well, Sigma) for 10 min, and absorbance at 490 nm was determined by ELISA reader (BioTek Instruments, Winooski, Vt., USA). Each cell viability assay was performed in quadruplicate and repeated three times. Data are expressed as means $\pm$ SEM, and differences were analyzed by Student's *t* test.

Flow-Cytometric Assay

Cells were seeded on 24-well plates and cultured under regular conditions until they reached the desired confluence. Mouse immune serum diluted 1:200 was added and cells were incubated at 37°C and 5% CO_2 for 48 h. Thereafter, cells were trypsinized and washed with PBS and fixed in ice-cold 70% ethanol overnight. Cells were then treated with RNase at 37°C and labeled with DNA-Prep Reagent (Sangon) for 20 min at RT. Finally, cells were analyzed by an Elite ESP-type flow cytometer (Beckman Coulter, Fullerton, Calif., USA) and cell ratios in the different phases were calculated.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from frozen lung tissue using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's protocol. Up to 5 μ g of total RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Clontech). The cDNAs were used as templates for quantitative real-time polymerase chain reaction (qRT-PCR). For MUC5AC, the primers were 5'-TCCGGCCTCATCTTCTCC-3' (forward) and 5'-ACTTGGGCACTGGTGCTG-3' (reverse); for GM-CSF, they were 5'-GGAGCATGTGAATGC-3' (forward) and 5'-GGATGACAAGCAGAAAGTC-3' (reverse), and for β -actin 5'-CTC-

CATCCTGGCCTCGCTGT-3' (forward) and 5'-GCTGTCACCTTCACCGTTCC-3' (reverse), which were used to amplify β -actin as a control. The qRT-PCR system contained 5 μ l SsoFast™ EvaGreen Supermix (Bio-Rad, Hercules, Calif., USA), 1 μ l of cDNA (diluted 1:50) and 2 μ l of each forward and reverse primer (1 μ M) to a final volume of 10 μ l. The following PCR procedure was employed: 94°C for 4 min and 20 s, 55°C for 30 s, and 72°C for 20 s, and 2 s for plate reading (35 cycles; melt curve from 65 to 95°C). β -Actin was used as a control to normalize gene expression. Three independent experiments were performed. The data obtained were calculated by $2^{-\Delta\Delta C_t}$ and statistical analysis was conducted as described previously [29], followed by an unpaired *t* test.

Periodic Acid-Schiff Staining

Periodic acid-Schiff (PAS) staining was performed according to a previous report [30]. Briefly, cells were collected and fixed in 10% formalin for 24 h at 4°C, and embedded in paraffin and cut into 5- μ m thin sections. Then, sections were immersed in periodic acid oxidation liquid for 20 min, washed with PBS twice, and stained with the Schiff reagent (GENMED, Shanghai, China) in the dark for 10 min. Harris hematoxylin was used to label nuclei for 2 min. Photomicrographs were obtained using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Immunocytochemistry

Cells were collected and fixed with 4% formaldehyde for 20 min, and subsequently washed with PBS. Cells were permeabilized with PBS containing 0.5% Triton X-100 (Sangon) for 10 min at 37°C. After washing with PBS, cells were incubated in 30% H_2O_2 :methanol (1:50) for 30 min to inactivate endogenous enzymes and then washed with PBS. Cells were immersed in 0.01 M citric acid solution (pH 6.0) and heated for 10 min in a microwave oven. After cooling of the cells, they were washed with PBS twice. The cells were then incubated with antigen repair liquid for 8 min followed by blocking with normal goat serum (Sangon) for 1 h at RT. Next, rabbit anti-GM-CSF antibody (diluted 1:1,000, Santa Cruz Biotechnology) as the primary antibody was added and incubated overnight at 4°C. After washing with PBS three times, cells were incubated with the biotin-labeled goat anti-rabbit antibody (1:3,000; Boster) for 2 h at 37°C. The streptavidin-biotin complex (Abcam) was added and incubated for 1 h at 37°C. After washing with PBS, cells were visualized with diaminobenzidine (Shanghai Haoran, Shanghai, China) and photographed using a fluorescence microscope (Leica Microsystem).

Statistical Analysis

All experiments were performed independently at least three times. Differences between groups were analyzed by Student's *t* test. A value of $p < 0.01$ was considered statistically significant.

Results

Generation and Identification of the NCI-H292/hCLCA1 Cell Line

The NCI-H292 cell line is a kind of AEC from lung adenocarcinoma that is often used as a goblet cell model in vitro owing to its biological activity that is extremely

Fig. 1. Confirmation of pIRES2-EGFP/hCLCA1 recombinant plasmid transfection into NCI-H292 cells. **a** Untransfected NCI-H292 cells. **b** NCI-H292 cells transfected with the pIRES2-EGFP/hCLCA1 construct. Magnification: $\times 200$. **c** Western blotting to

examine the expression of hCLCA1 in NCI-H292 cell lines. Lane 1 = NCI-H292 cells; lane 2 = NCI-H292/hCLCA1 cells; β -actin was used as a control.

Fig. 2. Comparison of the ultrastructure of NCI-H292 and NCI-H292/hCLCA1 cells. **a, b** NCI-H292 cells: microvilli (**a**) and mitochondria (**b**). **c–e** NCI-H292/hCLCA1 cells: karyokinesis (**c**), rough endoplasmic reticulum (**d**) and mitochondria (**e**). Magnification: $\times 4,000$.

similar to goblet cells [8]. As the NCI-H292 cell line does not express hCLCA1, an expression vector for hCLCA1 (pIRES2-EGFP/hCLCA1) was introduced into the NCI-H292 cell line to allow constitutive hCLCA1 expression. We successfully obtained transfected cells by neomycin (G418) selection. Next, to confirm that the recombinant vectors were expressed in the cells, GFP fluorescence was assessed using a fluorescence microscope. As expected, the transfected cells showed strong green fluorescence (fig. 1a) compared to the control (fig. 1b), indicating that the vectors constructed were successfully expressed in the cells. Furthermore, Western blotting was performed to test whether hCLCA1 protein was expressed in the transfected cells, and a specific 120-kDa band was detected in the NCI-H292/hCLCA1 cell extracts while no bands were detected in the control cells (fig. 1c). Taken

together, we successfully established a goblet cell model in vitro via overexpression of hCLCA1 in NCI-H292 cells.

Comparison of Ultrastructural Changes in NCI-H292 Cells and NCI-H292/hCLCA1 Cells

To determine whether transfection of pIRES2-EGFP/hCLCA1 influenced the intracellular morphology of NCI-H292 cells, we examined ultrastructural changes using a transmission electron microscope. The results showed that clear nucleolus and normal euchromatin were present in control cells, rough endoplasmic reticulum was rare, and microvilli were abundant on the outside of the cell membrane (fig. 2a). In addition, some bubble mitochondria were distributed at the edge of the cell membrane but lacked cristae (fig. 2b). In contrast, NCI-

H292/hCLCA1 cells exhibited different characteristics. Hyperactive nuclear division was observed and numerous translucent secretory granules were present near the outer membrane (fig. 2c). Rough endoplasmic reticulum covered with polyribosomes was arranged in a concentric lamellar shape (fig. 2d). Mitochondria were short and rectangular in shape, and rich in cristae and elementary particles (fig. 2e). These results demonstrated that NCI-H292/hCLCA1 cells exhibited hyperactive biosynthesis and proliferation, implying that hCLCA1 promotes cell biosynthesis and cell proliferation.

hCLCA1 DNA Vaccine Effectively Inhibits Proliferation of NCI-H292/hCLCA1 Cells

As the ultrastructure of NCI-H292/hCLCA1 cells showed hyperactive biosynthesis and proliferation, we next performed MTT and flow-cytometric assays to investigate the proliferation and cell cycle of NCI-H292/hCLCA1 cells, and also the effects of hCLCA1 DNA vaccine challenge. The results showed that cell viability of the H1 group was significantly higher than that in the control group C1 ($p < 0.01$). When these cells were treated with immune serum (group H2), cell viability was efficiently downregulated to normal levels ($p < 0.01$; fig. 3a).

To further investigate hCLCA1 DNA vaccine treatment effects on the cell cycle, we performed flow cytometry. Compared to the C1 group (G1: 63.07%, G2: 6.27%, and S: 30.66%), in H1 the proportion of cells in G1 (55.85%) and G2 phase (0.38%) was significantly decreased and notably increased (43.77%) in S phase ($p < 0.01$; fig. 3b). When exposed to immune serum, in the H2 group the proportion of cells in G1 phase (62.00%) was apparently upregulated compared to the H1 group (G1, 55.85%), but in S phase it was lower (31.69%) than in the H1 group (43.77%; $p < 0.01$). Each phase of the C1, C2 and H2 groups has no apparent changes. Overall, these results demonstrate that overexpression of hCLCA1 results in NCI-H292 cell hyperplasia and that the hCLCA1 DNA vaccine could effectively suppress these effects.

hCLCA1 DNA Vaccine Suppresses hCLCA1 Expression in NCI-H292/hCLCA1 Cells

To assess the effects of hCLCA1 DNA vaccine on the expression of hCLCA1 in different cell treatment groups, Western blot analysis was performed to analyze the protein level of hCLCA1. Results showed that hCLCA1 protein was rarely detected in groups C1 and C2 of NCI-H292 cells. The expression of hCLCA1 was highly upregulated in the H1 group, which received control serum

Fig. 3. The effects of overexpression of hCLCA1 and hCLCA1 DNA vaccine on cell proliferation and cell cycle. **a** Comparison of the proliferative activities of cells in all groups by MTT assay. Forty-eight hours after serum treatment, proliferative activity was assayed following the MTT procedure. Each cell viability assay was performed in quintuplicate and repeated three times. **b** Comparison of the cell cycle in all groups. Forty-eight hours after serum treatment, cells were harvested and the proportion of cells in each phase of the cell cycle was assayed in each study group. β -Actin was used as a control. All experiments were performed independently three times. Means $\pm$ SEM; ** $p < 0.01$ (Student's t test).

Fig. 4. Effect of hCLCA1 DNA vaccine on hCLCA1 expression. Western blot was performed to analyze protein expression of hCLCA1 in the different cell treatment groups. β -Actin was used as a control.

Fig. 5. Examination of mucin production levels in cell treatment groups. **a** Determination of MUC5AC transcriptional levels in cell groups using real-time PCR. Cells were treated with serum and incubated for 48 h. After treatment, cells were harvested for real-time PCR to measure MUC5AC levels. All experiments were independently performed at least three times. Means $\pm$ SEM; ** $p < 0.01$ (Student's *t* test). **b** Examination of MUC5AC protein levels by Western blot. β -Actin was used as a control. Bar = 20 μ m; magnification: $\times 400$. **c** Examination of mucin protein levels by PAS staining. Purple staining indicates mucins. Blue staining indicates nuclei stained with Harris hematoxylin. Bar = 20 μ m; magnification: $\times 400$.

treatment, and was significantly inhibited in the H2 group, which received immune serum against hCLCA1 (fig. 4).

hCLCA1 DNA Vaccine Suppresses Mucus Hypersecretion in NCI-H292/hCLCA1 Cells

Goblet cell hyperplasia resulted in mucus hypersecretion. In order to examine the changes in mucus production levels with or without hCLCA1 DNA vaccine challenge, we performed qRT-PCR, Western blot, and PAS staining to investigate both mRNA and protein levels of mucins, respectively. MUC5AC is a predominant component of mucus produced by goblet cells, and thus it can reflect the secretion levels of mucus production. As shown in the results, the mRNA level of MUC5AC was significantly higher in the H1 than the control group (fig. 5a). When treated with immune serum, mRNA levels of MUC5AC were effectively downregulated. Furthermore, Western blot was performed to determine protein expression of MUC5AC. Results showed that MUC5AC proteins exhibited similar effects, in accordance with the mRNA level following immune serum treatment (fig. 5b). Moreover, we performed PAS staining in which mucus glycoconjugates and glycoproteins were indicated in

bright purple. These results showed that H1 showed highly bright purple whereas C1 showed slight purple staining, implying that NCI-H292/hCLCA1 cells produced more mucus than control cells. When treated with immune serum, the degree of staining was significantly decreased in the H2 group, indicating that the hCLCA1 DNA vaccine could suppress mucus production (fig. 5c). Taken as a whole, overexpression of hCLCA1 induced increased mucus production and this could be efficiently blocked by hCLCA1 DNA vaccine.

hCLCA1 DNA Vaccine Inhibits GM-CSF Expression in NCI-H292/hCLCA1 Cells

GM-CSF is a critical cytokine in airway inflammation and is highly abundant in bronchial alveolar lavage fluid, lung tissues, and serum of asthmatic patients [31, 32]. GM-CSF was reported to be tightly associated with asthma and is mainly produced by AECs. However, it remains unclear whether goblet cells express GM-CSF. To analyze the expression level of GM-CSF in NCI-H292/hCLCA1 cells, we performed RT-PCR and immunocytochemistry. RT-PCR results demonstrated that mRNA levels of GM-CSF were significantly induced in the H1 compared to the C1 group (fig. 6a). On the other hand,

Fig. 6. Analysis of GM-CSF expression in cell treatment groups. **a** Real-time PCR showing GM-CSF expression in the cell treatment groups. All experiments were independently performed at least three times. Means $\pm$ SEM. ** $p < 0.01$ (Student's *t* test). **b** Immunocytochemistry showing GM-CSF protein levels in the cell treatment groups. Brown staining indicates GM-CSF protein. Blue staining indicates nuclei stained with Harris hematoxylin. Bar = 20 μ m; magnification: $\times 400$.

when treated with immune serum, H2 showed decreased expression levels of GM-CSF. To confirm this result, immunocytochemistry was carried out to analyze the protein level of GM-CSF. Compared to the control, H1 showed deep brown staining, which is considered strongly positive. When treated with immune serum, H2 showed mild brown staining, almost at the normal level (fig. 6b). These results showed that GM-CSF could be highly induced by overexpression of hCLCA1, and antagonizing hCLCA1 by immune serum was sufficient to prevent GM-CSF expression.

Discussion

In this study, we demonstrated that overexpression of hCLCA1 caused increased cell proliferation and highly upregulated MUC5AC and GM-CSF expression in goblet cells, which were the basis of airway mucus hypersecretion, airway hyperresponsiveness, and inflammatory responses in asthma. Thus, inhibiting hCLCA1 expression and/or antagonizing hCLCA1 activity are potential therapeutic strategies for asthma.

The NCI-H292 cell line is a kind of AEC from lung adenocarcinoma that has been often used as a goblet cell model in vitro [8, 33, 34]. Recently, Alevy et al. [35] established a cell line (NCI-H292-rtTA-CLCA1 cells) under the control of a doxycycline-inducible promoter system which could express hCLCA1 stably and was sufficient for mucin gene expression. Although they found some hCLCA1 mRNA expression at baseline in NCI-H292 cells, NCI-H292 cells still represented a useful model for mucin study in vitro. In our study, we rarely detected hCLCA1 protein expression in NCI-H292 cells by Western blot analysis, indicating that hCLCA1 protein was rarely expressed in these cells. In recent years, it has become clear that AECs play an important role in airway inflammation, and they produce a broad range of mediators, including many kinds of cytokines, chemokines, lipids, and peptides, which in turn influence cellular processes of the airway epithelium [36]. Evidence accumulated that numerous cytokines and chemokines secreted by inflammatory cells could increase the goblet cell number by mucous metaplasia and goblet cell hyperplasia [37–39]. Mucous metaplasia implies a change in the cell phenotype, while hyperplasia suggests cell pro-

liferation as the mechanism for the increase in goblet cell numbers. In allergic asthma, IL-9 and IL-13 produced by T-helper 2 (Th2) lymphocytes significantly enhanced goblet cell hyperplasia [40–42]. There are also studies demonstrating that hCLCA1 and mCLCA3 levels are upregulated by Th2 cytokines, including IL-4, IL-9, and IL-13 during asthma [26]. Thus, it is clear that hCLCA1 regulated by Th2 cytokines is tightly associated with goblet cell hyperplasia in the pathology of asthma. As reported, the hallmark of goblet cells in asthma was hyperplasia [1], which was responsible for mucus overproduction in asthma. In the present study, NCI-H292 cells represented a high proliferative rate by overexpression of hCLCA1, which mimicked the state of goblet cells in asthma *in vitro*. The results showed that NCI-H292/hCLCA1 cells possess hyperactive nuclear division, and numerous translucent secretory granules existed near the outer membrane. Also, rough endoplasmic reticulum decorated with polyribosomes, and mitochondrial cristae and elementary particles were highly increased. These ultrastructural changes indicated that the transfected cells demonstrated hyperactive biosynthesis and proliferation by hCLCA1 overexpression, suggesting that hCLCA1 could promote cell biosynthesis and cell proliferation. Furthermore, MTT assays demonstrated that the transfected cells had higher cell viability than untransfected cells, and hCLCA1 DNA vaccine could effectively downregulate the cell viability increase. However, the underlying mechanism of hCLCA1 stimulation of cell proliferation remains unclear and needs further investigation.

In a mouse model of allergic asthma, mucus hypersecretion was significantly decreased by inhibition of mCLCA3 [8], and administration of respiratory syncytial virus, which usually causes bronchiolitis, could induce mCLCA3 and MUC5AC expression dramatically [43]. In the present study, we showed that MUC5AC expression could be induced by hCLCA1, which was consistent with the report by Nakanishi et al. [8]. MUC5AC was proposed to be regulated by various signaling pathways, including cyclic AMP response element binding protein, epithelial growth factor receptor (EGFR), mitogen-activated protein kinases (MAPK), and nuclear factor κ B pathways [44]. Recent studies indicated that EGFR could induce MUC5AC expression by activating the Ras/Raf/MEK1,2/ERK1,2 cascade in goblet cells [45, 46]. EGFR has been implicated in critical functions regulating several cellular progresses, including cell growth, cell differentiation, and cell repair of AECs [47], which has been already found to be highly upregulated in AECs of asthma patients [45,

48]. Th2 cytokines were found to promote goblet cell numbers and regulate MUC5AC expression by MAPK and phosphatidylinositol 3-kinase regulation [49]. It was reported that IL-13 induced both MUC5AC and hCLCA1 expression in goblet cell hyperplasia, implying the important role of the two molecules in goblet cell hyperplasia [50]. It is possible that hCLCA1 might be acting as a signaling molecule involved in the above signaling pathways regulating MUC5AC expression.

GM-CSF plays a crucial role in airway inflammation produced mainly by AECs, which can induce eosinophil infiltration [32, 51]. A specific antibody recognizing GM-CSF was reported to inhibit airway hyperresponsiveness and airway mucus cell proliferation in an allergic asthma mouse model [52]. GM-CSF could be induced by tumor necrosis factor- α , IL-1 α and platelet-activating factor, which is involved in the MKK3,6/p38MAPK signaling pathway in human bronchial epithelial cells [53]. Recently, it has been reported that environmental fungi prompt the production of GM-CSF related to human asthma. Thus, GM-CSF plays an important role in the development of asthma [54]. In the present study, we found that hCLCA1 could trigger GM-CSF expression in goblet cells, and hCLCA1 vaccine effectively inhibited the production of GM-CSF, which implies that hCLCA1 may function as an upstream signaling molecule in the regulation of asthma development.

hCLCA1 and mCLCA3 in HEK293 cells could be detected in the extracellular medium and the N-terminal cleavage submits of these two proteins could also be detected in bronchoalveolar lavage fluid of asthma patients and in an allergic asthma mouse model [20]. Secreted hCLCA1 and mCLCA3 had zinc-dependent autoproteolytic activity which might cleave other proteins [23, 24]. Thus, CLCA proteins are no longer thought to function as chloride channels and might serve crucial roles in calcium signaling. The secreted CLCA that is cleaved into peptides may activate a signal in the epithelium to induce mucus production. It is possible that hCLCA1 is responsible for activating or regulating a signaling pathway involved in MUC5AC and GM-CSF expression regulated by Th2 cytokines. However, these underlying mechanisms need to be further studied.

To date, treatment of asthma is still in its infancy. More recently, a combination of dexamethasone and itraconazole could effectively inhibit fungus-exacerbated asthma [55]. Glucocorticoids, the traditional asthma drugs, can ameliorate asthma to a certain extent, but it cannot treat the underlying pathological causes of asthma [56]. Therefore, it is necessary to seek the initial location and critical

gene targets for asthma therapy. Thus, considering the role of hCLCA1 in asthma pathology, hCLCA1 may serve as a potential therapeutic target. Although specific inhibitors targeting the subtype of CLCA protein are currently not available, DNA vaccination provides an attractive technique for protecting an organism against disease through activation of the immune system and antibody production. A variety of DNA vaccines has been developed in human clinical trials against various infectious diseases and cancer because of its advantages over traditional vaccines. Allergens and pathogens that induce asthma are the current focus of DNA vaccine therapies. However, few of the pathological molecules related to asthma, such as hCLCA1, have been investigated to date. Following the injection of a genetically engineered DNA molecule, the body will develop an adaptive immune response, including antibodies, Th cells and cytotoxic T lymphocytes as well as an innate immune response [57]. In the present study, we demonstrated that hCLCA1 antibodies in the mouse serum might inhibit the functions of hCLCA1 by antibody antagonism or enhancement of immune functions. However, the precise mechanism requires further elaboration. Given the functions of hCLCA1 in airway inflammation, one can speculate that hCLCA1 overexpression might activate im-

mune cells contributing to airway inflammation through an unknown pathway which in turn promotes hCLCA1 expression. Thus, inhibition of hCLCA1 by hCLCA1 DNA vaccine might interrupt the harmful loop which attenuates airway inflammation in asthma. Here we found that the antiserum against hCLCA1 obtained upon immunizing mice with an hCLCA1 DNA vaccine effectively inhibited cell proliferation and expression of MUC5AC as well as GM-CSF in goblet cells.

In conclusion, we established an in vitro goblet cell model by introducing hCLCA1 into the NCI-H292 cell line. Using this cell model, an hCLCA1 DNA vaccine was found to significantly suppress cell proliferation and mucin expression in goblet cells. Thus, an hCLCA1 DNA vaccine is expected to show good effects on the control of goblet cell hyperplasia and mucus hypersecretion, and represents a potential treatment for asthma.

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